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Begin the article with a short summary.

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Only standard abbreviations are acceptable. If a new or coined abbreviation is used, it must be defined when first mentioned.

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The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through November 30, 1976.

LETTER

Uncommon Side Effects of Adriamycin (NSC-123127)

We read with interest the article by Rothberg et al (1) and wish to report another adriamycin reaction that has not been generally reported. Although the literature is replete with reports of the classic manifestations of adriamycin toxicity (2–4), there is a paucity of information on allergic reactions to the drug or its effect on previously irradiated areas. We have observed these reactions in 60 children with a variety of solid tumors who received adriamycin alone or in combination with other agents, usually vincristine.[1] The dose of adriamycin generally used was 60 mg/m^2 given by intravenous (iv) push. When given in combination, adriamycin was customarily preceded by vincristine, and normal saline flush was administered before adriamycin was given.

There were 18 episodes of allergic reactions observed in 13 of 60 children receiving adriamycin. These reactions were localized in all but one patient who also had anaphylaxis (nonfatal) shortly after a localized reaction had ensued.

The local reactions consisted of an erythematous streak along the vein (similar to an angiitis) proximal to the injection site and involving about 6–8 inches of the vein. The reaction was associated with pruritus, patchy erythema, and/or urticaria along the vein. It was generally transient in nature, lasting about 30 minutes, and disappeared spontaneously or after the iv administration of 1 mg/kg of diphenhydramine hydrochloride. The skin rash became apparent during or immediately after injection. Five patients reacted with the first dose and others experienced a reaction as late as the eighth dose. In three patients who reacted with the first dose, no further reactions occurred with subsequent doses. Three patients had consecutive repeated reactions. One of these patients was subsequently premedicated iv with diphenhydramine hydrochloride in an attempt to prevent a recurrence of the allergic reactions. However, this prophylactic measure was not uniformly successful because the patient manifested the same reactions on a few occasions despite premedication. The two other patients were removed from the therapy for various reasons unrelated to adriamycin.

Vincristine given iv prior to adriamycin was thought to be a factor that predisposed the patients to allergic reactions, but because similar reactions were also noted in patients receiving adriamycin alone, this theory was discounted. The saline flush was also unlikely to be incriminated because of the absence of reactions in other children in whom the same solution was utilized to flush various iv chemotherapeutic agents.

Skin reactions reminiscent of those commonly observed with actinomycin D (NSC-3053) radiation-recall phenomena were seen in two children. In one girl who was irradiated for mandibular fibrosarcoma, a cutaneous and mucosal reaction (the latter only on the irradiated side) lasting for about 7 days occurred with the first two doses of adriamycin, approximately 1 month after completion of the radiation therapy. This was associated with local pain and irritation. The second episode was less intense and there was no recurrence with subsequent doses. In another patient who received irradiation to the leg, pain, swelling, erythema, and skin breakdown developed in the irradiated area with the first two doses of adriamycin. The first reaction occurred 1 week after completion of the irradiation.

For the most part, it appears that these reactions are of no major detrimental consequence except perhaps in an occasional patient in whom a localized reaction may be the herald of a potentially catastrophic anaphylaxis.

[1] Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

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August 22, 1974

COMMENTARY

The Private Practice of Medical Oncology: An Analysis [1,2]

Michael B. Mosher, Robert J. Taub, and Melvin Avedon[3,4,5]

SUMMARY

In an effort to assess the current status of the management of cancer in a metropolitan community, a detailed analysis of an adult medical oncology practice in Los Angeles will be presented. All patients seen by a three-man group of internists-oncologists in calendar year 1973 will be analyzed. Demographic data will be offered, comparing the oncology patients with those seen as part of the general medical practice within the same office. Patterns of referral will be discussed with particular reference to which types of cancer are actually being referred to private oncologists. Tumor types will be analyzed for specific treatment received with some estimates of response rates, complications, and mortality. Included will be a discussion of the attitudes in private practice that may vary from those in clinical research centers. The feasibility, frequency, and general usefulness of investigational agents will be explored. Suggestions relative to the improvement of the overall physical and emotional support of cancer patients and their families will be offered.

[Cancer Chemother Rep 58:759–767, 1974]

This report will detail the practice of a group of three medical oncologists in Los Angeles, California. All patients seen in calendar year 1973 are included. Only adult patients are seen, and each is treated by all three physicians over the course of therapy. The intention of this presentation is to determine what types of cancer are being seen in private practice, in what frequency each type is encountered, and the overall results of any chemotherapy received, including investigational agents. Practical as well as philosophical differences that exist between oncology practiced in cancer research centers and that practiced in the private community will be commented on.

In terms of serving as a guide to the structure of oncology training programs, perhaps this paper will act as a companion piece to Dr. David Prager's analysis of a hematology practice presented in 1972 (1).

[1] Received June 19, 1974; accepted June 20, 1974.

[2] The compounds used in these studies were supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, National Cancer Institute. Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

[3] Division of Oncology, Cedars-Sinai Medical Center, and Divisions of Surgical and Medical Oncology, University of California, Los Angeles.

[4] The authors gratefully acknowledge the help of Ms. Jerene Baglin, MA, in the treatment of our patients, and the assistance of our office staff in the collection of the data.

[5] *Reprint requests to:* Michael B. Mosher, MD, 8733 Beverly Blvd, Los Angeles, Calif 90048.

PATIENT MATERIAL

General Information

Most patients are referred to us by their internist or surgeon. Only occasionally is a

patient referred from another patient and even more rarely is he sufficiently aware of the existence of oncology as a specialty to request such consultation on his own. The bulk of patients are referred when obvious recurrent disease has been found or is strongly suspected. We are not often asked to see a patient in order to direct presurgery staging or routine postsurgery followup. On the other hand, rarely are we asked to see a patient who is clearly in the terminal stages of his illness. There is a growing trend, however, toward involvement of the oncologist earlier in the course of the disease.

In 1973 our office saw 410 patients with malignant disease, including old and new patients. Of these, 107 (25%) were seen in consultation only, whereas all others (303 patients) either were, or became, our patients for followup management. In contrast, of the 30 patients seen for non-neoplastic hematologic problems, 25 (83%) were seen for one-time consultation only (*see* table 1).

TABLE 1.—Breakdown of patients seen in 1973

	No. of patients	
	Oncology	Hematology
Started year with us	150	2
Acquired in 1973	153	3
Seen in consultation only	107	25
	410	30

Patient Distribution

Figure 1 shows the age and sex distribution of our cancer patients as compared to 231 general medical patients seen in the same office in 1973. As might be expected, the cancer patients fell into a much narrower and older age range, heavily centered on the age range of 50–80 years. If one eliminates breast and genital-organ malignancies, the sex distribution is still 54% female, a trend also true of the general medical patients, where 57% were female.

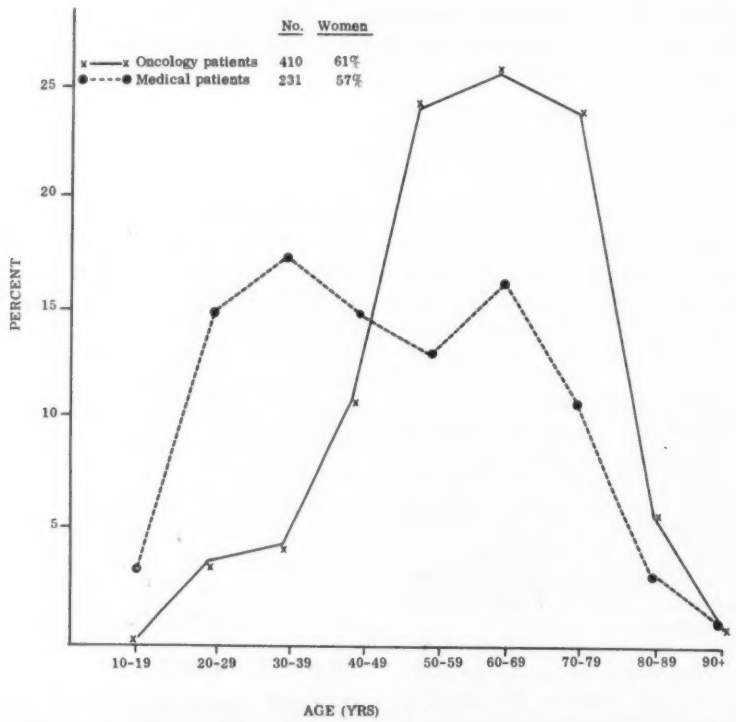

FIGURE 1.—Age and sex distribution of cancer patients as compared to general medical patients seen in the same year.

Disease Distribution

Table 2 gives the breakdown of our patients by specific histologic type of cancer. We saw more than 20 types of cancer, but 296 patients (72%) fell into just four types: breast, colon, lung, and hematologic malignancies. This is due, of course, both to the relative frequency of the first three types and the general knowledge among physicians that lymphomas are highly treatable by a qualified oncologist. Overall, nonhematologic malignancies ("solid tumors") represented 78% of the total. We saw eight patients with more than one malignancy; these are outlined in table 3.

TABLE 2.—Distribution of patients by tumor type

Histologic type	No. of patients
Breast	99
Hematologic	92
Lymphoma	51
Hodgkin's disease	12
Multiple myeloma	10
Chronic lymphocytic leukemia	8
Chronic myelogenous leukemia	7
Polycythemia vera	2
Acute myelogenous leukemia	1
Waldenström's disease	1
Colon	57
Lung	48
Uterus	13
Pancreas	12
Head and neck	12
Ovary	16
Sarcoma	10
Rhabdomyosarcoma	2
Liposarcoma	4
Osteogenic sarcoma	2
Kaposi's sarcoma	1
Leiomyosarcoma	1
Gastric	8
Renal	7
Prostate	5
Melanoma	4
Brain	5
Bladder	7
Testicle	2
Thymoma	2
Thyroid	2
Adrenal	1
Esophagus	2
Adenocarcinoma, unknown primary	6
	410

RESULTS OF CHEMOTHERAPY

At a given point in time, some of our patients are not receiving chemotherapy but instead may be undergoing radiation therapy, taking hormonal therapy, or not receiving any cancer-specific therapy at all. Most parenteral chemotherapy is given by a state-certified medical assistant, but procedures such as thoracentesis, paracentesis, and bone marrow aspiration are done only by the physician. We average about three such procedures per week. A physician's assistant would probably be of great usefulness in this regard, but these assistants are not licensed to perform such procedures in California.

In the following sections, complete remission is defined as the alleviation of all symptoms secondary to cancer, as well as complete objective regression of measurable disease. Partial response is defined as significant subjective improvement, usually associated with some degree of objective response. A fair number of our patients did not have measurable disease that could be followed; in many patients all that was demonstrable was lack of progression, eg, a recurrent pleural effusion that did not recur once chemotherapy was started. Therefore, we elected to include in the partial-responder group any patients whose disease did not progress while receiving therapy, if their disease had been clearly progressing prior to treatment. This type of response was worthwhile to the patient and was somewhat gratifying to us, but it would certainly not meet the standard requirements for response in a clinical research study. Additionally, most of these partial responses lasted only a few months.

TABLE 3.—Types of multiple cancers seen in 1973

Patient No.	Sites of disease
1	Breast, thyroid, pancreas
2	Breast, ovary, lung
3	Breast, brain
4	Breast, parotid
5	Breast, lung
6	Breast, liposarcoma
7	Testicle, lung, stomach
8	Colon, lymphoma (both progressive simultaneously)

The next sections of this paper will present the results of chemotherapy in the three most frequently treated neoplasms—non-Hodgkin's lymphoma, breast cancer, and colon cancer.

Non-Hodgkin's Lymphoma

Our lymphoma patients, all over the age of 40 and almost all in stages III or IV, are treated with weekly intravenous (iv) doses of vincristine and cyclophosphamide and 10-day courses of prednisone given orally each month. Once remission is achieved, this therapy is stopped after 8–12 weeks and the remission is maintained with monthly courses of chlorambucil and prednisone given orally. This allows regrowth of hair and a marked decrease in the number of office visits a patient needs, permitting the return by the patient to a more nearly normal existence.

As can be seen in table 4, our complete remission rate is 79%, and 20 of 22 complete remitters are still in remission. It should be kept in mind that our patients are not rehospitalized to thoroughly evaluate completeness of remission. Remission duration is counted from the end of the initial intensive period of iv treatment, usually 8 weeks. It does not seem that we have sacrificed anything in terms of remission duration by this method, compared to, admittedly, historical controls. Further, without breaking down this data by specific histologic categories, real comparisons are impossible.

Breast Cancer

Of the 99 patients with breast cancer, there was one man. When nonhormonal therapy is indicated, we treat all patients with multiple-agent chemotherapy, including 5-fluorouracil (5-FU), methotrexate, vincristine, cyclophosphamide, and, usually, prednisone, in a modification of the Cooper regimen. Again, in an attempt to improve the quality of our patients' lives, we maintain remissions using a single agent (usually 5-FU or cyclophosphamide) after a 10–12-week remission-induction period. Table 5 demonstrates that our complete

TABLE 4.—Response* of lymphoma to cyclophosphamide + vincristine + prednisone

No. of patients treated	No. of patients with—			Duration of remission (mos)			
				Still in remission		No longer in remission and originally had—	
	CR	PR	NR	CR	PR	CR	PR
28	22	4	2	2+,2+,4+, 8+,9+, 9+,9+, 10+,10+, 13+,16+, 16+,16+, 18+,18+, 19+,20+, 24+,50+	7+, 23+	13,17	2,6

* CR = complete remission; PR = partial response; NR = no response.

remission rate is 57%, with another 34% deriving some subjective, generally short-lived, benefit. Complete remission can be very difficult to define, however, because we have the problem of when to call the healing of a bone lesion or the resolution of an abnormal bone scan really complete. The median duration of remission is about 9 months, which is not very different from that seen when remissions are maintained on the full multiple-agent program by other therapists. We would suggest a study comparing multiple-drug versus single-agent remission maintenance, with reinduction using multiple agents in the latter group. In some patients we have also added a hormonal agent during the period of remission in the hope of prolonging remission. This combined chemotherapy and hormonal therapy is uncontrolled and unevaluable in a clinical study sense.

Colon Cancer

Our initial treatment of metastatic colon carcinoma is with weekly iv 5-FU, often alternating with oral 5-FU after the establishment of a stable dose. Table 6 shows the poor results obtained. No complete remissions were seen and only 19% of the patients showed partial remissions, rarely lasting longer than 4 months. Also shown is our experience with weekly 5-FU given to patients with poor prognostic lesions. Thus far, 50% have recurred, and in four of the remaining eight patients less than 1 year has elapsed since surgery; thus, our own feeling is that, at least in a weekly schedule, 5-FU is of no value in this situation and as a general rule we have not been recommending its use.

In 5-FU failures we have often tried a combination of daily oral melphalan and weekly iv methotrexate; we saw partial responses in 25% of the patients, all of brief duration.

Investigational Therapies

With the cooperation of Dr. Stephen Carter and the now-famous Mrs. Gladstone, we have had access to investigational agents.

Table 7 details the results with CCNU given in a dose of 130 mg/m² orally every 6 weeks. We found it to be a highly disappointing drug, with only six brief responses in 29 patients.

Table 8 shows the results with adriamycin, usually given in a dose of 60 mg/m² iv every 3 weeks. This dosage regimen seems to have very tolerable toxicity, except for total alopecia. Though the drug seems to be active in breast cancer, responses were brief. We have used these investigational drugs only as single

TABLE 5.—Response* of breast carcinoma to combination therapy with 5-FU, cyclophosphamide, methotrexate, vincristine, and prednisone

No. of patients treated	No. of patients with—			Median duration of CR (mos)
	CR	PR	NR	
35	20	12	3	9

* CR = complete remission; PR = partial response; NC = no response.

TABLE 6.—Response* of colon carcinoma to chemotherapy

Treatment	No. of patients treated	No. of patients with—				
		CR	PR	NR	NED	Recurrence
5-FU, weekly iv	26	0	5 (19%)	21		
Melphalan orally + methotrexate iv	12	0	3 (25%)	9		
5-FU prophylactically	16	—	—	—	8†	8

* CR = complete remission; PR = partial response; NR = no response; NED = no evidence of disease.

† Four patients had NED for <1 year.

agents, and all data are reported to the National Cancer Institute (NCI).

Table 9 details the results of FU-COM therapy (5-FU, cyclophosphamide, vincristine [Oncovin], and methotrexate) in a variety of disease types. All four drugs are given iv once per week. This combination did show activity in halting progression in lung cancer and in obtaining actual regressions in bladder carcinoma and adenoid-cystic carcinoma of the head and neck region (one patient).

Since one of us (Dr. Mosher) trained with Dr. Bertino at Yale University School of Medicine, we have also given an assortment of patients gram-doses of methotrexate followed by leucovorin "rescue." The most interesting of these was a man with progressive epidermoid cancer of the lung whose disease ceased progression for 5 months; at that time the drug had to be discontinued because of the development of probably drug-related, but reversible, renal failure.

TABLE 7.—Results of treatment with CCNU (100–130 mg/m² orally)

Disease type*	No. of patients treated	No. of patients with—†		
		CR	PR	NR
Colon	10	0	2	8
Ovary	5	0	0	5
Lung				
Alveolar cell	3	0	1	2
Adenocarcinoma	1	0	1	0
Testicle	1	0	0	1
Renal	2	0	0	2
Brain	1	0	1	0
Hodgkin's disease	1	0	0	1
Gallbladder	1	0	0	1
Thymoma	1	0	0	1
Pancreas	2	0	0	2
Adenocarcinoma	1	0	1	0
	29	0	6	23

* Seven patients with gliomas were unevaluable.
† CR = complete remission; PR = partial response; NR = no response.

TABLE 8.—Results* of treatment with adriamycin (60 mg/m² iv q3wks)

Disease type	No. of patients treated	No. of patients with—		
		CR	PR	NR
Breast	6	0	4	2
Sarcoma	1	0	0	1
Ovary	1	0	0	1
Kidney (transitional cell)	1	0	0	1
Histiocytic lymphoma	1	1	0	0
	10	1	4	5

* CR = complete remission; PR = partial response; NR = no response.

TABLE 9.—Results* of treatment with cyclophosphamide, vincristine, methotrexate, and 5-FU (FU-COM) weekly iv

Disease type	No. of patients treated	No. of patients with— CR	PR	NR
Bladder	5	1	2	2
Lung				
Adenocarcinoma	4	1	3	0
Epidermoid	1	0	1	0
Alveolar cell	3	0	2	1
Ovary	2	0	1	1
Adenoid-cystic carcinoma, head and neck	2	1	0	1
	17	3	9	5

* CR = complete remission; PR = partial response; NR = no response.

Our experience with hepatic artery ligation is included in a report by Dr. Frank Sparks of UCLA presented at the meetings of the American Society of Clinical Oncology, March 1974. Clearly, private practice does lend itself to participation in investigational procedures.

Mortality

Mortality data are presented in table 10. Of 150 patients already under our care on January 1, 1973, 56 (37%) died by the end of that year. Of 153 who came under our care during 1973, 43 (28%) died by the end of that year, producing a 33% overall mortality rate. Specifically, twice a week (99 times in all) we saw one of our patients die. Four of these deaths were drug related.

Psychological Support

What psychological support is available for our patients? In general, we have found private psychiatry, if for no other reason than financial, not practical. Additionally, though there is no shortage of psychiatrists in neighboring Beverly Hills, few of them have an interest in, or facility for, treating cancer patients.

TABLE 10.—Mortality data for 1973

	Total No. seen	No. who died in 1973/%
Old patients	150	56/37
New patients	153	43/28
	303	99/33

Recently, several organized group meetings of cancer patients have been developed, sponsored either by the American Cancer Society or Mt. Sinai Hospital. Our initial experience with these has been excellent and we feel this is an approach well worth pursuing. The local American Cancer Society chapter has also developed "Cancervive," a program of home visits of cancer patients to newly diagnosed cancer patients, with some encouraging results thus far. Our office conducts weekly group meetings with patients' families, where they can share common anxieties, depression, and anger. We have long felt that the families are often neglected during this illness and need much support and counselling. Ideally, an oncology office should have a full-time psychiatric counselor (such as a trained social worker or nurse) to provide really complete care to the cancer patient. Currently, we employ on a part-time basis a bright, empathetic, former teacher to give brief additional help to the patients at each visit.

DISCUSSION

Having had experience both in private practice and at major cancer treatment centers

(NCI and Yale), we would like to make a few observations on the differences observed in being an oncologist in such different settings.

First, the patients themselves have different attitudes. At the two centers, patients tended to accept all research procedures and plans without question. They usually had acknowledged before arriving that they were to be part of ongoing research projects. In contrast, our patients frequently express the need not to be the proverbial "guinea pig," and even the concept of randomization is often intolerable. It also appears that our patients are far more easily seduced into nonstandard methods of treatment such as that offered in the Laetrile clinics in Mexico or by other physicians in the Southern California area (who shall remain unnamed). It is the rare patient at the NCI or Yale who seeks another opinion, but in our practice we receive such requests about once a month. On the other hand, we in turn are just as often consulted by patients under the care of other oncologists or university cancer centers in the area. Lastly, the referring internist or surgeon occasionally tries to retain control of the therapeutic situation, a practice we try to discourage because it is often confusing and detrimental to the patient. Such a situation would be very unusual at the NCI.

Since we function in community hospitals, the practicing medical oncologist has a far greater opportunity to educate his fellow nononcologic physicians on a day-to-day basis than does the physician in a center. Convincing the surgeon of the necessity of pre-operative staging of breast and lung cancers, or suggesting radiation therapy as the treatment for oat cell carcinoma, are a few examples of the opportunities available to make an impact on the general quality of cancer management. We feel a responsibility for changing out-dated concepts of cancer care and for educating the nononcologist about the biology of the disease. Physicians' misconceptions are a bigger problem to us than patients' misconceptions, since the former are much more difficult to change.

Although at both the NCI and Yale there was very great and sincere concern for each patient as an individual, there was also an overriding sense of commitment to learn as much as possible from each patient about his disease. There was a real feeling of responsibility to the cancer patient of 1980. In practice, our passion lies in worrying almost entirely about the cancer patient under our care in 1974. Any information gained that may be of value to the art of oncology is of course prized but is in reality only incidental to our main pursuit. We should reemphasize, as has been shown, that we can and do use investigational and innovative treatment methods, and we feed data to protocol groups. However, there are several handicaps we operate under in this regard. We do not have the luxury of refusing to treat patients because of prior treatment attempts or because of the lack of measurable disease to follow. It is very difficult to truly randomize patients, especially if one arm of the study involves no treatment, such as in the prophylactic 5-FU study. We have no financial support to pay staff whose primary function would be to monitor studies and collect data. On the other hand, since we are not tightly committed to a protocol in most cases, we have far greater flexibility in individualizing the treatment to the person.

We look to the institutions for well-designed, adequately controlled studies and hope that they look to us to apply their advances to the huge bulk of patients who will never find their way there. Thus, though we see our respective roles as entirely distinct, they are quite complementary. There is no point to progress if it is not widely applied.

Should we refer all patients to major centers? Most patients simply do not want to travel long distances from home in order to participate in a study which may not offer them, as individuals, a much better chance of response than that obtainable in their own community. We think that most of the referrals to oncology centers should come from the areas without oncologists. However, acute leukemia represents an exception. Our attitude has been to offer referral to the patient with acute myeloblastic leukemia, explaining the possible benefits of laminar air flow rooms and white blood cell transfusions. We would very strongly urge, if not insist, that the patient

with acute lymphoblastic leukemia be treated only at a center specializing in that disease. Patients and their families occasionally request consultation with an institution, which we then strongly encourage, both for our education and their peace of mind.

An important question to be considered is whether current training programs adequately prepare a physician for the actual practice of medical oncology. In terms of supplying basic knowledge about cancer, concepts of treatment, and details about the drugs, training seems excellent. However, there is a clear-cut disproportion in cancer types studied in fellowships versus those seen in practice. The former tend to deal in diseases within which tumor responses are expected, particularly hematologic malignancies and breast cancer. But at least 50% of the patients seen in our practice fell into other disease types. The overall management of these others was learned more in practice than in training. For example, we saw only 12 patients with Hodgkin's disease (2% of the practice), a disease strongly emphasized in most training programs. Table 11 shows our collected response rates in the major types of cancers. If hematologic malignancies and breast cancer are eliminated we are left with a 6% complete remission rate and a 32% partial response rate, and most of these remissions are quite brief. Therefore, for a great number of patients the most we as medical oncologists really have to offer is counselling to the patient and her family so that they can make the best possible adjustment to their situation. Though we all spend much time and effort with complex and intensive chemotherapy regimens hunting for the occasional regression, to the patient with cancer of the lung, pancreas, colon, kidney, stomach, uterus, etc, what we really have to offer is some symptomatic short-term palliation, a bit of hope, and the judgment to know when to stop chemotherapizing and when to help provide for the best possible death.

LIST OF COMPOUNDS

Adriamycin: NSC-123127

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

Chlorambucil: NSC-3088; CAS reg. No. 305-03-3; butyric acid, 4-[*p*-[bis(2-chloroethyl)amino]phenyl]-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU

Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-; Alkeran

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

REFERENCE

1. PRAGER D. An analysis of hematologic disorders presenting in the private practice of hematology. Blood 40:568–573, 1972.

TABLE 11.—Response* to chemotherapy in major treatment groups

Disease type	No. of patients treated	No. of patients with— CR	PR (includes no progression)	NR
Lymphoma	28	22	4	2
Breast	35	20	12	3
Colon	26	0	5	21
Lung	17	0	12	5
Gastric	4	0	1	3
Pancreas	8	0	3	5
Ovary	13	3	2	8
Bladder	5	1	2	2
	136	46 (33%)	41 (30%)	49 (37%)
Excluding lymphoma and breast	73	4 (6%)	25 (32%)	44 (62%)

* CR = complete remission; PR = partial response; NR = no response.

Biologic Activity of Some Adriamycin (NSC-123127) Derivatives [1,2]

L. Lenaz,[3,4,5] A. Necco,[3,4] T. Dasdia,[3,4] and A. Di Marco [3,6,7]

SUMMARY

This report describes the activity of a group of new adriamycin derivatives on HeLa cells and a spectrum of experimental tumors (ascites and solid Sarcoma 180, Gross leukemia, and mammary carcinoma). All derivatives obtained by esterification of the 14-hydroxylic group of the saturated ring of adriamycinone showed marked antitumor activity, generally more pronounced for compounds with an aliphatic side chain. Under our experimental conditions, adriamycin-14-octanoate appears to be the most promising of the adriamycin derivatives tested in view of its lower toxicity, especially when used against Gross leukemia.

[Cancer Chemother Rep 58:769–776, 1974]

The antineoplastic activity of adriamycin in animals and man has been extensively described. The antitumor effects seen in experimental systems (1–3) were reflected by the action of the drug in patients with acute leukemia (4,5) and different kinds of solid tumors (4,6). In addition to its therapeutic effects however, adriamycin causes major toxic side effects (such as myelodepression and cardiotoxicity) (4–6) which reduce its clinical usefulness in short treatment courses. Because of the significant clinical activity of adriamycin, many compounds chemically related to this anthracycline antibiotic were synthesized by Prof. Arcamone and his co-workers at the Department of Chemistry, Farmitalia Research Institute, Milan, Italy. These compounds were studied in our laboratory in the hope of finding agents with a better therapeutic index than the parent compound.

The antitumor effects (7) as well as data on the structure-activity relationships (8) of some daunorubicin derivatives have been previously described. The present report describes in vitro and in vivo studies on the cytotoxic and antitumor activity of a new group of adriamycin derivatives.

MATERIALS AND METHODS

In Vitro Studies

HeLa cells were cultured in monolayers in Eagle's basal medium supplemented with 10% calf serum in 75-cm² plastic flasks. The mitotic index was determined 8 hours after drug treatment by the score of 3000 cells/sample. For determination of the effects on proliferative activity, 40×10^4 cells were seeded in 60-mm plastic Falcon dishes. After 22 hours the cells were exposed to the drugs and treatment was continued for 8 hours, the drugs then be-

[1] Received Feb 6, 1973; revised July 19, 1974; accepted Aug 22, 1974.

[2] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

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[4] On leave from Farmitalia Research Institute, Milan, Italy.

[5] *Present address:* Farmitalia Research Institute.

[6] The authors are indebted to Mrs. T. Comisso and Mrs. F. Meloni for their excellent technical assistance.

[7] *Reprint requests to:* Professor A. Di Marco, Division of Experimental Oncology B, Istituto Nazionale per lo Studio e la Cura dei Tumori, Via Venezian 1, Milan 20133, Italy.

ing removed and replaced by normal medium. After 40 hours cells were counted in a hemocytometer. Treatment was performed with various dilutions covering at least a 1-log range.

In Vivo Studies

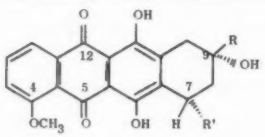
Sarcoma 180 (S180) ascites cells were inoculated intraperitoneally (ip) into Ha/ICR mice of the CD1 line (Charles River Breeding Laboratory, Paris, France), and then bred in our laboratory (1×10^6 cells/mouse). Solid S180 was implanted subcutaneously (sc) by trocar in the flank of CD1 mice according to standard methods (1). In the case of mammary carcinoma, 20×10^6 cells were inoculated sc as previously described (3) in C3H/He Dp inbred mice. Gross leukemia, originally induced by infection of newborn mice with the Gross leukemia virus, was transplanted in C3H/He Dp mice by intravenous (iv) inoculation of 2.5×10^6 cells from an homogenate of spleens and lymph nodes of leukemic mice. On the 11th day after solid S180 implantation the animals were killed and the tumors excised and weighed. The T/C ratio (tumor weight index) was used for evaluating the antitumor activity.

For the other transplanted tumors, the activity of the compounds under study was assessed by determining the increase in average survival time. When measured, the volume of solid tumors was evaluated by the formula $a \times b$, a and b being the minimum and maximum diameters respectively.

All compounds studied were chromatographically pure (98%) and were dissolved in Ringer solution with the exception of adriamycin-14-octanoate and adriamycin-14-benzoate, which were dissolved in 1% dimethylsulfoxide (DMSO) in distilled H_2O. Drugs were administered ip or iv in a volume of 10 ml/kg of body weight. Appropriate groups of animals treated with an equal volume of the solvent were used as controls.

TABLE 1.—Chemical structures of adriamycin derivatives

General structure



R' = daunosamine

Compound	Molecular weight	R=
Daunorubicin • HCl	563.5	$-CO \cdot CH_3$
Adriamycin • HCl	580.0	$-CO \cdot CH_2OH$
14-Bromo-daunomycin • HCl	643.0	$-CO \cdot CHBr$
Adriamycin-oxime • HCl	594.5	$-C = NOH \cdot CH_2OH$
Adriamycin-14-acetate • HCl	623.0	$-CO \cdot CH_2 \cdot O \cdot CO \cdot CH_3$
Adriamycin-14-propionate • HCl	636.0	$-CO \cdot CH_2 \cdot O \cdot CO \cdot CH_2CH_3$
Adriamycin-14-octanoate • HCl	706.2	$-CO \cdot CH_2 \cdot O \cdot CO \cdot (CH_2)_6 \cdot CH_3$
Adriamycin-14-benzoate • HCl	684.1	$-CO \cdot CH_2 \cdot O \cdot CO$ + benzene ring
Adriamycin-14-nicotinate • HCl	684.0	$-CO \cdot CH_2 \cdot O \cdot CO$ + pyridine ring (N)
Adriamycin-14-(phenylacetate) • HCl	698.0	$-CO \cdot CH_2 \cdot O \cdot CO \cdot CH_2$ + benzene ring
Adriamycin-14-(1-naphthaleneacetate) • HCl	748.1	$-CO \cdot CH_2 \cdot O \cdot CO \cdot CH_2$ + naphthalene ring

RESULTS

The chemical structures of the adriamycin derivatives are shown in table 1. Of these compounds, adriamycin-14-octanoate and adriamycin-14-benzoate were soluble in organic solvents (ethanol or DMSO), adriamycin-14-(1-naphthaleneacetate) was soluble at 0.5% in H_2O, and all other drugs were freely soluble in water.

In Vitro Studies

Preliminary screening was carried out on HeLa cells (table 2). While 14-bromo-daunomycin and adriamycin-oxime were relatively ineffective in inhibiting mitotic activity, adriamycin-14-acetate was as active as adriamycin, and the other derivatives displayed an activity intermediate between that of adriamycin and daunorubicin. None of the derivatives showed higher activity in inhibiting cell proliferation than the parent compounds.

In Vivo Studies

Preliminary in vivo screening was carried out on S180 ascites tumor (table 3). Of all the derivatives tested, adriamycin-14-acetate

TABLE 2.—Effects of adriamycin derivatives on HeLa cells

Compound	ID50 on mitotic activity* (μg/ml)	ID50 on cell proliferation* (μg/ml)
Daunorubicin	0.030 (0.003–0.057)	0.10 (0.07–0.13)
Adriamycin	0.140 (0.064–0.216)	0.17 (0.14–0.20)
14-Bromo-daunomycin	> 2	2.21 (1.60–2.82)
Adriamycin-14-acetate	0.270 (0.120–0.400)	0.99 (0.60–1.39)
Adriamycin-oxime	> 2	> 2
Adriamycin-14-octanoate	0.061 (0.046–0.076)	1.12 (0.72–1.51)
Adriamycin-14-benzoate	0.079 (0.058–0.100)	1.26 (0.87–1.65)
Adriamycin-14-nicotinate	0.045 (0.027–0.063)	0.99 (0.71–1.26)
Adriamycin-14-propionate	0.057 (0.040–0.074)	0.71 (0.47–0.94)
Adriamycin-14-(phenylacetate)	0.046 (0.028–0.064)	1.36 (0.87–1.85)
Adriamycin-14-(1-naphthaleneacetate)	0.064 (0.058–0.070)	1.24 (0.84–1.65)

*Values in parentheses represent 95% confidence limits.

TABLE 3.—Activity in ascites S180*

Compound	Dose range tested (mg/kg)	Optimal dose (mg/kg)	Average survival time† (days ± 9.2)	No. of tumor-free 50-day survivors/ No. treated
Control	—	—	13.4 ± 0.6	0
Daunorubicin	0.125–2	0.25	29.7 ± 1.9	6/11
Adriamycin	0.125–2	0.50	30.4 ± 2.1	8/11
14-Bromo-daunomycin	1–8	2.0	27.9 ± 2.8	3/10
Adriamycin-14-acetate	0.125–8	2.0	36.3 ± 2.0	7/10
Adriamycin-oxime	1–8	4.0	26.3 ± 3.9	1/10
Adriamycin-14-octanoate	0.25–8	2.0	38.0 ± 3.1	5/10
Adriamycin-14-benzoate	0.5–8	1.0	33.3 ± 5.1	2/10
Adriamycin-14-nicotinate	0.5–8	2.0	26.0 ± 3.9	1/10
Adriamycin-14-propionate	0.5–8	2.0	24.8 ± 2.2	1/10
Adriamycin-14-(phenylacetate)	0.5–8	1.0	23.1 ± 1.9	2/10
Adriamycin-14-(1-naphthaleneacetate)	0.5–8	4.0	29.5 ± 4.0	4/10

*Mice were inoculated with 10^6 ascites cells on Day 0 and treated with a single ip dose on Day 1.
†Long-term survivors are excluded from the computation of average survival times.

and adriamycin-14-octanoate were the most active compounds, inducing an increase in average survival time and a number of tumor-free long-term survivors similar to those induced by daunorubicin and adriamycin.

In this system however, no compound showed activity significantly higher than that of the parent compounds. When tested in solid S180 (table 4) adriamycin-oxime was almost totally inactive, while all other compounds tested displayed significant activity, generally similar to that of adriamycin.

Most of the compounds tested in Gross leukemia (table 5) displayed significant activity (ie, increase in lifespan of 40% or more versus untreated controls). In particular, adriamycin-14-octanoate induced a greater increase in survival time and a larger number of long-term survivors compared to adriamycin (fig 1). The difference in survival time between

TABLE 4.—Activity in solid S180*

Compound	Maximal tolerated dose (mg/kg/day)	Average tumor weight (g ± SE)	Inhibition (%)
Control	—	4.5 ± 0.15	—
Daunorubicin	3.25	2.2 ± 0.3	50
Adriamycin	2.5	1.4 ± 0.2	69
14-Bromo-daunomycin	10.0	2.7 ± 0.6	39
Adriamycin-14-acetate	2.5	1.6 ± 0.5	64
Adriamycin-oxime	10.0	4.4 ± 1.4	3
Adriamycin-14-octanoate	3.5	1.2 ± 0.1	73
Adriamycin-14-benzoate	3.25	1.4 ± 0.2	68
Adriamycin-14-nicotinate	3.25	1.6 ± 0.2	64
Adriamycin-14-propionate	3.25	1.5 ± 0.1	67
Adriamycin-14-(phenylacetate)	3.25	1.6 ± 0.2	62
Adriamycin-14-(1-naphthaleneacetate)	4.0	1.4 ± 0.2	69

*Mice were treated iv on Days 1–8. On Day 11 the tumors were excised and weighed.

TABLE 5.—Activity in Gross leukemia*

Compound	Optimal dose (mg/kg/day)	Average survival time		No. of long-term 60-day survivors/ No. treated
		Days ± SE	%T/C	
Control	—	10.3 ± 0.2	—	—
Daunorubicin	3.25	19.0 ± 0.5	170	0/10
Adriamycin	2.5	21.0 ± 0.2	204	1/30
14-Bromo-daunomycin	7.6	11.1 ± 1.1	108	0/10
Adriamycin-14-acetate	2.5	18.8 ± 0.7	183	0/10
Adriamycin-14-octanoate	4.0	26.6 ± 2.7	258	3/30
Adriamycin-14-benzoate	2.5	17.4 ± 1.1	169	0/10
Adriamycin-14-nicotinate	4.0	16.1 ± 0.5	156	0/10
Adriamycin-14-propionate	3.25	20.0 ± 3.8	194	0/10
Adriamycin-14-(phenylacetate)	4.0	20.6 ± 2.1	200	0/10
Adriamycin 14-(1-naphthaleneacetate)	4.0	17.1 ± 0.6	166	0/10

*Mice received an iv inoculation of 2.5×10^6 leukemic cells/mouse. Treatment was performed iv on Days 1–5.

TABLE 6.—Activity in established mammary carcinoma*

Treatment	Dose (mg/kg/day)	Tumor volume 30 days after beginning of treatment (cm^3 ± SE)†	Average survival time (days ± SE)
Control	—	8.60 ± 0.69	52.9 ± 6.1
Daunorubicin	3.25	1.85 ± 0.34	58.8 ± 5.1
Adriamycin	2.5	1.37 ± 0.22	75.0 ± 4.1
Adriamycin-14-acetate	2.7	1.79 ± 0.28	90.2 ± 5.9
Adriamycin-14-octanoate	3.1	1.09 ± 0.32	96.8 ± 6.6
Adriamycin-14-benzoate	3.1	2.04 ± 0.16	71.6 ± 7.5
Adriamycin-14-nicotinate	2.9	2.02 ± 0.53	74.1 ± 7.6
Adriamycin-14-propionate	2.7	1.59 ± 0.18	87.8 ± 4.9
Adriamycin-14-(phenylacetate)	3.0	1.72 ± 0.31	75.2 ± 5.2
Adriamycin-14-(1-naphthaleneacetate)	3.2	2.35 ± 0.30	73.9 ± 6.8

*Treatment was started 3 weeks after the sc inoculation of 20×10^6 cells/mouse. Mice were treated with 2 cycles of 5 daily iv doses with a 2-day interval between the cycles. The doses used correspond to the maximum tolerated doses for the schedule of treatment used.

†The average tumor volume at the beginning of treatment was 0.45 ± 0.02 cm^3.

mice treated with optimal doses of adriamycin and mice treated with optimal doses of adriamycin-14-octanoate is statistically significant ($P<0.05$). Adriamycin esters were tested in established mammary carcinoma and the results are shown in table 6. In this case treatment was started 3 weeks after transplant when the average tumor diameter was approximately 0.5 cm^3. All derivatives showed marked activity in inhibiting tumor growth; the best results, both in inhibiting tumor growth and in increasing the survival time of the treated animals, were obtained with adriamycin-14-octanoate. These effects however, were not statistically significant compared with those obtained with adriamycin. Adriamycin-14-ace-

TABLE 7.—Activity on transplanted mammary carcinoma*

Compound	Dose (mg/kg/day)	No. of mice with tumors/ No. treated	Day of tumor appearance	Toxic deaths: No. of deaths/ No. of mice treated	Toxic deaths: Average day of death
Control	—	10/10	21	0/10	—
Adriamycin	2	5/10	98.8	0/10	—
	2.25	4/10	110.2	2/10	96.5
	2.5	1/10	106	6/10	90.0
Adriamycin-14-acetate	2	5/10	90.8	0/10	
	2.25	2/10	96.5	0/10	
	2.5	1/10	108	1/10	198.0
Adriamycin-14-octanoate	2.5	2/10	87.0	0/10	
	2.8	2/10	110.0	1/10	157.0
	3.2	0/10	—	10/10	84.5
Adriamycin-14-(1-naphthaleneacetate)	3	10/10	26.3	0/10	—
	4	7/10	41.6	1/10	115.0
	5	4/10	51.5	5/10	91.0

*Iv treatments were given q2d, Days 1–11 and 17–27. Animals were observed for 240 days.

tate and adriamycin-14-octanoate were selected for further studies and were tested on the early transplanted mammary carcinoma. Treatment with these agents, as with adriamycin, resulted in a remarkable number of tumor-free long-term survivors at nontoxic doses (table 7), while treatment with adriamycin-14-(1-naphthaleneacetate) resulted in less pronounced effects. Pathologic examination showed that the delayed deaths observed in tumor-free animals can probably be ascribed to intercurrent infections.

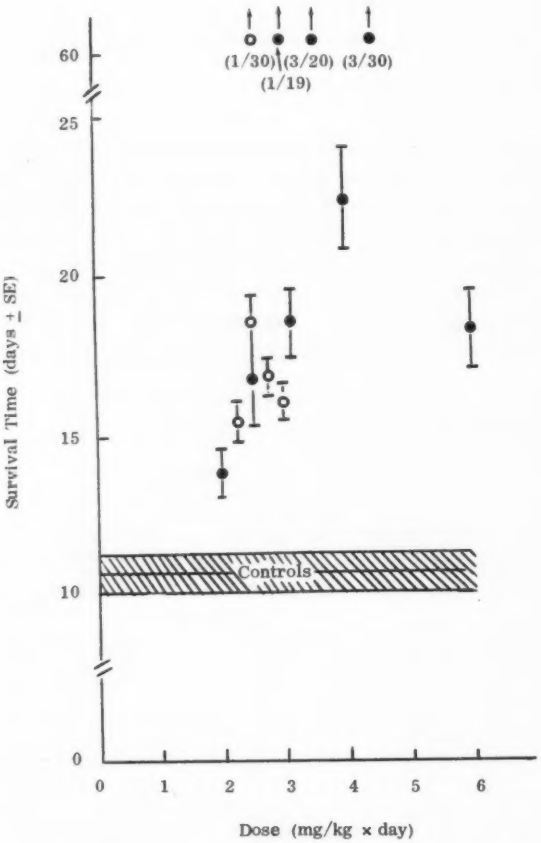


FIGURE 1.—Effects of adriamycin (open circles) and adriamycin-14-octanoate (filled circles) on survival time of C3H mice bearing transplanted Gross leukemia. Symbols at 60 days indicate long-term survivors/No. of treated animals. Animals were treated iv daily for 5 successive days.

In all systems, treatment with 1% DMSO at a dose of 10 mg/kg did not result in any significant differences compared to the saline-treated controls. In a control experiment, the effects of adriamycin dissolved in 1% DMSO or saline were compared in the transplantable Gross leukemia. The results reported in table 8 show that different solvents did not induce any significant differences in the antileukemic effects of adriamycin. We have also attempted to administer adriamycin-14-octanoate in vehicles other than DMSO, ie, fine suspensions in 5% ethanol. No significant difference was observed when the drug was administered iv in the two vehicles for each of 5 successive days to mice bearing Gross leukemia.

DISCUSSION

The data presented in this paper indicate that the derivatives of adriamycin, obtained by esterification of the hydroxylic group in position 14 of the saturated ring of the aglycone, retain marked antitumor activity. All derivatives in this group display marked activity on HeLa cells in vitro, suggesting that metabolic modifications outside the target cells are not required in order to effect activity. It is conceivable however, that metabolic modification of adriamycin may take place within the cells by nonspecific esterases. Preliminary data (Lenaz L, and Di Fronzo G, unpublished results) suggest that this is the case for adriamycin-14-octanoate.

Although no definite conclusion can be drawn due to the limited number of derivatives, compounds esterified with an aliphatic acid chain appeared to have antitumor activity higher than those esterified with an aromatic acid. In particular, adriamycin-14-octanoate appeared to be highly active both in a rapidly growing system such as the transplanted Gross leukemia and in a slowly growing system such as mammary carcinoma. When treatment with adriamycin-14-octanoate or adriamycin-14-acetate was carried out in this latter system shortly after transplant, the appearance of the tumor was prevented in 80%–90% of the animals at nontoxic doses, while adriamycin at tolerated doses prevented tumor growth in 50% of the animals. Adriamycin-14-octanoate, al-

TABLE 8.—Effects of solvent (1% DMSO) on the activity of adriamycin on Gross leukemia*

Treatment	Dose (mg/kg/day)	Average survival time (days ± SE)
No drug treatment		9.6 ± 0.4
(1% DMSO only)		9.4 ± 0.5
Adriamycin dissolved	2.0	20.7 ± 1.8 (1)
in water	2.5	17.4 ± 0.7 (2)
	3.0	16.4 ± 0.4
Adriamycin dissolved	2.0	20.3 ± 3.9 (1) NS†
in 1% DMSO	2.5	21.3 ± 2.8 (2) NS
	3.0	17.7 ± 1.1

*Mice were inoculated iv with 2.5×10^6 leukemia cells and treated iv on Days 1–5.
†NS = not significant.

though very active in Gross leukemia and S180, did not prevent tumor appearance in the animals treated with nontoxic doses.

It should also be stated that in our experiments treatment with adriamycin against established mammary carcinoma was far more effective than previously reported by other workers (9). These discrepancies can be attributed to the different route and schedule of treatment used. Sandberg and Goldin (9), for example, used ip administration, and the optimal schedule against ascitic L1210 leukemia was every 3 hours × 8 for 1 day. As stated in the literature (10), ip as compared to iv treatment results in increased toxicity, and a slow-growing tumor with a growth fraction of approximately 50% (such as the mouse mammary carcinoma [11]) requires schedules of treatment different from those used for the rapidly growing L1210 leukemia.

The toxicity of adriamycin-14-octanoate also appears lower than that of adriamycin, the maximal tolerated dose for eight daily iv treatments being 3.5 and 2.5 mg/kg/day respectively. These differences in antitumor effect and toxicity could be tentatively related to the different solubilities of the two compounds, adriamycin-14-octanoate being practically insoluble in water. Because different affinity to the lipophilic cellular structures may lead to different pharmacokinetic properties, studies on the distribution of this drug are currently in progress in this laboratory (Lenaz L, and Di Fronzo G, unpublished results).

Preliminary data indicate that adriamycin-14-octanoate is excreted more rapidly than adriamycin and indeed has a different pharmacokinetic behavior; in particular, it can be seen that in the mouse, the compound, while taken up more rapidly by liver and lungs, is concentrated to a smaller extent in the heart. These data, as well as further studies in other experimental systems such as the Moloney virus-induced rhabdomyosarcoma and the Lewis lung carcinoma, will be reported separately.

LIST OF COMPOUNDS

Adriamycin: NSC-123127

Adriamycin-14-acetate: NSC-149581; CAS reg. No. 41962-27-0; adriamycin, 14-acetate, hydrochloride

Adriamycin-14-benzoate: NSC-149585; CAS reg. No. 41962-28-1; adriamycin, 14-benzoate, hydrochloride

Adriamycin-14-(1-naphthaleneacetate): NSC-203768; 1-naphthaleneacetic acid, (2S-*cis*)-2-[4-[(3-amino-2,3,6-trideoxy-α-L-lyxo-hexapyranosyl)oxyl]-1,2,3,4,6,11-hexahydro-2,5,12-trihydroxy-7-methoxy-6,11-dioxo-2-naphthacenyl]-2-oxoethyl ester

Adriamycin-14-nicotinate: NSC-149583; adriamycin, 14-nicotinate, monohydrochloride

Adriamycin-14-octanoate: NSC-149584; adriamycin, 14-octanoate, hydrochloride

Adriamycin-oxime: NSC-143491; daunomycin, 3-oxime, monohydrochloride, sesquihydrate

Adriamycin-14-(phenylacetate): NSC-149582; CAS reg. No. 41962-30-5; adriamycin, 14-(phenylacetate), hydrochloride

Adriamycin-14-propionate: NSC-149586; CAS reg. No. 41962-29-2; adriamycin, 14-propionate, hydrochloride

Daunorubicin: NSC-83142; CAS reg. No. 23541-50-6; daunomycin (NSC-82151)

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Correlation of 5-Fluorouracil (NSC-19893) Distribution in Rodents With Toxicity and Chemotherapy in Man [1,2,3]

Robert H. Liss and Marjory Chadwick [4,5]

SUMMARY

The distribution of 5-fluorouracil (5-FU)-derived C^{14} radioactivity has been studied in BDF_1 mice bearing 6-day solid L1210 lymphocytic leukemia and in normal Fischer rats by a quantitative densitometric whole-body radioautographic technique. Radioactivity was widely distributed throughout the tissues between 2 and 96 hours after a single intravenous dose of 5-FU-2-C^{14} at 200 mg/kg (600 mg/m^2) to mice and 125 mg/kg (600 mg/m^2) to rats. Radioactivity accumulated in several tissues including the mucosal layer of the gastrointestinal tract, bone marrow, and cerebellum, each a site of 5-FU-related toxicity in man. Comparison of densitometrically and radiochemically determined radioactivity concentrations in tissues demonstrated the usefulness of the radioautographic technique in detecting and quantitating the specific localization of radioactivity within organs such as the brain. The distribution and persistence of 5-FU-derived radioactivity in rodent tissues was correlated with that of 5-FU nucleotides including the postulated active metabolite, 5-fluoro-2′-deoxyuridine-5′-monophosphate, and with the sites of toxic effects and choice of dose schedule in man. This study demonstrates the value of the quantitative whole-body radioautographic technique in preliminary preclinical drug disposition studies and serves as a model system for aiding in the prediction of toxicity and therapy regimens in man.

[Cancer Chemother Rep 58:777–786, 1974]

5-Fluorouracil (5-FU) is a synthetic pyrimidine that is used in the clinical management of tumors of the breast, colon, and rectum (1). 5-FU is believed to exert its major cytotoxic effect by inhibiting DNA synthesis (2,3) through its active form, 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP), which competitively inhibits thymidylate synthetase and thus, thymidylic acid formation (4–6). Clinical toxic effects associated with 5-FU therapy include gastrointestinal damage (1), bone marrow suppression (1), and neurotoxic effects (7,8). 5-FU produces similar gastrointestinal and hematologic toxic effects in rodents (9) and there are also similarities between the physiologic disposition of 5-FU in man and rodents (10–14).

The present investigation was undertaken (*a*) to relate the distribution of 5-FU-derived C^{14} radioactivity in tumor-bearing BDF_1 mice

[1] Received Feb 26, 1973; revised May 29, 1974; accepted June 21, 1974.

[2] Supported by contract NO1-CM-33727 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] 5-Fluorouracil: CAS reg. No. 51-21-8. Supplied by Drug Research and Development, DCT, NCI.

[4] Arthur D. Little, Inc., Cambridge, Mass.

[5] *Reprint requests to:* Robert H. Liss, PhD, Arthur D. Little, Inc., Acorn Park, Cambridge, Mass 02140.

and normal Fischer rats (as measured by a quantitative densitometric whole-body radioautographic technique) to data on the physiologic disposition of 5-FU in mice (determined by biochemical techniques) (10), and (*b*) to assess the relevance of this rodent model of 5-FU disposition to clinical therapy with this compound. A preliminary report of this study has been presented (15).

MATERIALS AND METHODS

5-FU-2-C^{14} (22 millicurie/mmol) was purchased from Calatomic, Los Angeles, California, and was shown chromatographically to be 96% pure.

Female BDF_1 mice weighing 20 g were implanted with 40-mg fragments of solid L1210 lymphocytic leukemia given subcutaneously in the axillary region. After 6 days, the mice were given single intravenous (iv) injections via the caudal vein of 5-FU-2-C^{14} in water, which was adjusted to pH 11 with KOH, at 200 mg/kg (600 mg/m^2). Mice killed at early time periods received approximately 300 microcurie/kg; those to be killed at late time periods received approximately 1000 microcurie/kg. Male Fischer rats weighing 80 g were given single iv injections via the caudal vein of 5-FU-2-C^{14} at 125 mg/kg (600 mg/m^2) and approximately 1700 microcurie/kg.

Groups of mice were frozen in liquid nitrogen at 2, 24, 48, 72, and 96 hours after dosage and rats were frozen at 12, 24, and 48 hours. All animals were subsequently processed for whole-body radioautography (16). The densitometric method for quantitation of the radioautograms has been previously described.[6]

For the physiologic disposition study, tumor-bearing BDF_1 mice were dosed as described above and groups were killed at various times after dosing, including 2, 24, 48, and 72 hours. Blood, liver, kidney, spleen, small intestine, bone marrow, brain, tumor, and urine samples were collected. The concentrations of total drug equivalents and of FdUMP were determined by methods previously described (10).

[6] Arthur D. Little, Inc., Cambridge, Mass. Annual report to the Chemotherapy Program, NCI. Section VII. Electron microscopy and radioautography studies, 1968.

RESULTS

Whole-Body Radioautography

The distribution pattern of 5-FU-derived C^{14} radioactivity was essentially the same for BDF_1 mice and for Fischer rats as observed by the radioautographic methods used in this experiment. Representative radioautograms are shown in figures 1–4.

Circulatory system.—The concentration of radioactivity in the blood at 2 hours after dosage was lower than in the body musculature, including the myocardium of the heart. Radioactivity persisted in the myocardium for 96 hours.

Lymphatic organs.—The spleen, lymph nodes, and thymus contained relatively high concentrations of radioactivity throughout the experiment. The red pulp of the spleen contained a fivefold greater concentration of radioactivity than the white pulp at 48 hours after dosage, and by 72 hours, the difference was tenfold.

Pancreas.—The pancreas contained relatively high concentrations of radioactivity throughout the experiment (figs 1 and 3).

Adrenal glands.—Radioactivity was present in the adrenal glands at all times. The adrenal cortex contained slightly higher concentrations than the medulla.

Reproductive organs.—Radioactivity was present in the testes and accessory sex glands at all times.

Nervous system.—Significant amounts of radioactivity were detected in the brain at all times. The cranial nerves, choroid plexus, and some spinal ganglia were imaged in radioautograms taken of both species. At 12 and 24 hours in the rat, the hypophysis contained a significant concentration of radioactivity. The cerebellum (especially the Purkinje and granular cell layers), cerebrum, corpus callosum, and hippocampus exhibited a significant accumulation of radioactivity (figs 1–3). Radioactivity was present in the eye at 2 hours, the highest concentration being in the lens and retina. By 96 hours, the concentration in the eye decreased, but both of these tissues were still imaged in the radioautograms.

Digestive system. Teeth contained a moder-

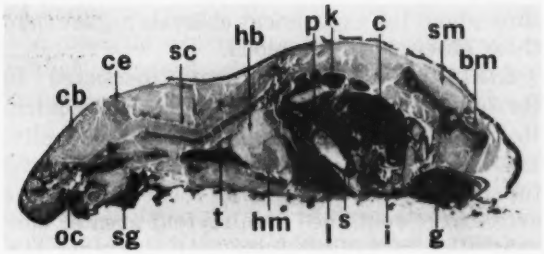

FIGURE 1.—Whole-body radioautogram of Fischer rat killed 12 hrs after a single iv dose of 5-FU-2-C^{14} at 125 mg/kg (600 mg/m^2) and approximately 1700 microcurie/kg. CB = cerebrum; CE = cerebellum; SC = spinal cord; HB = heart blood; P = pancreas; K = kidney; C = colon; SM = skeletal muscle; BM = bone marrow; G = gonad; I = intestine; S = stomach; L = liver; HM = heart muscle; T = thymus; SG = salivary gland; OC = oral cavity.

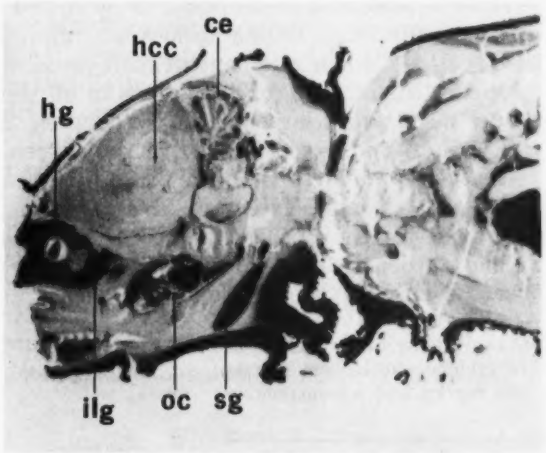

FIGURE 2.—Enlargement of portion of whole-body radioautogram of Fischer rat killed 24 hrs after a single iv dose of 5-FU-2-C^{14} at 125 mg/kg (600 mg/m^2) and approximately 1700 microcurie/kg. Note the deposition of drug-derived C^{14} radioactivity in cerebellar loci corresponding to Purkinje and granular cell layers and density throughout the cerebrum, especially the area of the hippocampus and corpus callosum. HG = Harderian gland; HCC = area of hippocampus and corpus callosum; CE = cerebellum; SG = salivary gland; OC = oral cavity; ILG = internal lacrimal gland.

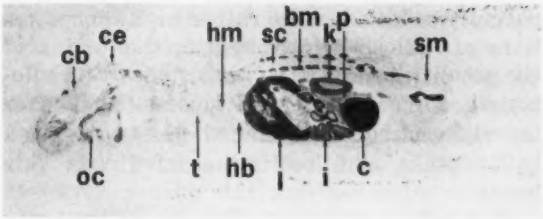

FIGURE 3.—Whole-body radioautogram of BDF$_1$ mouse killed 72 hrs after a single iv dose of 5-FU-2-C^{14} at 200 mg/kg (600 mg/m^2) and approximately 1000 microcurie/kg. At this later time period, much of the administered dose of 5-FU-derived C^{14} radioactivity has been excreted. Density in the radioautogram corresponds to sites of C^{14} retention. CB = cerebrum; CE = cerebellum; HM = heart muscle; SC = spinal cord; BM = bone marrow; K = kidney; P = pancreas; SM = skeletal muscle; C = colon; I = intestine; L = liver; HB = heart blood; T = thymus; OC = oral cavity.

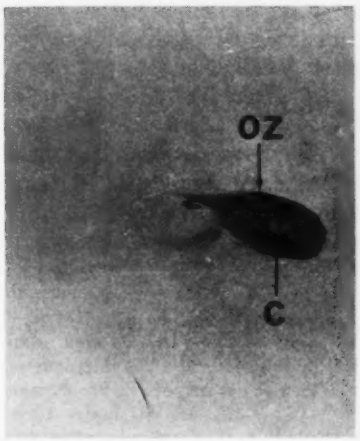

FIGURE 4.—Whole-body radioautogram of L1210 tumor from BDF$_1$ mouse killed 72 hrs after a single iv dose of 5-FU-2-C^{14} at 200 mg/kg (600 mg/m^2) and approximately 1000 microcurie/kg. Persistence of significant amounts of 5-FU-derived C^{14} radioactivity was observed in the L1210 tumor as late as 72 hrs after dosage. Typically, density was greatest in an outer zone (OZ) around a tumor center (C) with slight radioautographic density.

ate concentration of radioactivity in the pulpal and periodontal parts. The salivary glands, esophagus, and stomach also contained moderate concentrations of radioactivity throughout the experiment. Relatively high concentrations of radioactivity persisted in the liver for 96 hours; the highest concentration was observed at 2 hours. The bile and murine gallbladder contained a very high concentration

of radioactivity, and biliary secretion was apparently reflected in the rather high concentrations of radioactivity present in the lumina of the small intestine. The persistence of radioactivity for 96 hours in the mucosal layer after the radioactive contents had passed suggests both uptake and localization of C^{14} in this tissue.

Respiratory system.—Some radioactivity was detected in the nasal mucosa and nasal secretion. The parenchyma of the lung contained a higher concentration of radioactivity than the blood throughout the experiment.

Urinary organs.—Radioactivity concentrations were high in the kidney at 2 hours and were evenly distributed throughout the renal tissues. Between 24 and 96 hours however, the highest concentrations were found in the kidney cortex and cortico-medullary junction.

Skin.—Throughout the experiment radioactivity persisted in the integument of the skin and in the hair, as well as areas corresponding to the epidermis. Radioactivity was detected around the individual hairs in the hair follicles of the snout.

Hard tissues.—Radioactivity was not detectable in the hard tissues. However, it persisted in the bone marrow throughout the experiment.

Muscles.—Low concentrations of radioactivity persisted in the heart and skeletal muscle throughout the experiment at levels higher than those observed in the blood.

L1210 tumor.—Radioactivity persisted in the tumor for 96 hours; the highest concentration was observed at 2 hours. The radioactivity was not homogeneously distributed in the tumor mass. By 72 hours, for example, the outer layer contained a thirtyfold greater concentration of radioactivity than the inner layer (fig 4).

Physiologic Disposition

The physiologic disposition of 5-FU and its metabolites in mice, determined by biochemical techniques, has been reported in detail elsewhere (10). Only data necessary for comparison with the results of the radioautographic studies are included in this paper.

Table 1 summarizes the total drug equivalent concentrations in the tissues at various time periods when radioautographic studies were carried out. The concentrations in all the tissues decreased relatively rapidly between 2 and 24 hours and, except in the liver and spleen, decreased slowly over the next 2 days. In the spleen and to a lesser extent, the liver, the drug equivalent concentrations increased between 24 and 48 hours and although they decreased over the next 24 hours, the concentration in

TABLE 1.—Concentrations of total 5-FU equivalents in the tissues of BDF_1 mice bearing 6-day solid L1210 lymphocytic leukemia after 5-FU-2-C^{14} was administered iv at 200 mg/kg and approximately 300–1000 microcurie/kg

Pooled tissues of 5–10 mice	Concentrations (nmol/g)* of total 5-FU equivalents at—			
	2 hrs	24 hrs	48 hrs	72 hrs
Blood	160	4	3	2
Tumor	700	250	150	>110†
Bone marrow	>180†	23	12	9
Small intestine	520	110	70	43
Kidney	940	> 62†	65	51
Liver	1390	87	100	68
Spleen	900	94	200	130
Brain	92	22	14	10

*The concentrations of 5-FU equivalents in bone marrow were expressed in nmol/10^8 cells which approximates nmol/g.

†These data are the concentrations of acid-soluble 5-FU equivalents, as the concentrations of total 5-FU equivalents were not determined in these tissues at these time periods.

the spleen by 72 hours was still higher than at 24 hours.

A possible explanation for this observation is that advanced solid L1210 lymphocytic leukemia becomes systemic (17) and that by 8 days it has infiltrated the liver and spleen to an extent sufficient to affect drug disposition (10). In support of this hypothesis, the liver and spleen from normal animals at 48 and 72 hours after 5-FU was administered contained lower concentrations of drug equivalents than the same organs from tumor-bearing animals (10). Moreover, the drug equivalent concentrations in the spleen at 48 and 72 hours were comparable to those in the 8- and 9-day tumors. A probable explanation is that the infiltrating tumor cells arise from the outer layer of the solid tumor which (as reported above) contains a much higher concentration of drug equivalents than the center of the tumor. These data confirm the radioautographic observations that 5-FU equivalents persisted in the tissues and particularly in tumor cells for 72 hours after drug administration.

The FdUMP concentrations observed in the tissues when radioautographic studies were carried out are summarized in table 2. The postulated active metabolite persisted in the tissues at significant concentrations for 72 hours after 5-FU administration. The nucleotide is a potent competitive inhibitor of thymidylate synthetase. The reported K_m for deoxyuridine-5′-monophosphate is about 1.5×10^{-5} M, whereas the K_i is 5.2×10^{-8} M without preincubation and 3.6×10^{-9} M with preincubation of the enzyme with FdUMP (6,18).

In the tumor, the FdUMP concentration increased between 2 and 24 hours but decreased by 48 hours to one half the 24-hour concentration and remained at that level at 72 hours. However, during this time oncolytic drug action resulted in more than a 50% decrease in tumor weight, and the changes in FdUMP concentration, which were different from those observed in the other tissues, may reflect these changes in the tumor cell population (10). In the bone marrow and brain, the FdUMP concentration remained constant over the first 24 hours and then declined. In the small intestine, and to a lesser extent in the kidney, the FdUMP concentration remained relatively constant over 72 hours. In the liver and spleen the FdUMP concentration decreased between 2 and 24 hours. In the liver this concentration decreased between 24 and 48 hours to a level maintained at 72 hours, but in the spleen the FdUMP concentration increased at 48 hours and remained high at 72 hours. These atypical changes possibly resulted from the infiltration of dividing cells from the peripheral layer of the solid tumor into the spleen. In support of this hypothesis, the spleen from normal animals at 48 and 72 hours after 5-FU administration contained lower concentrations of FdUMP than the spleen from tumor-bearing animals (10). However, the liver from normal

TABLE 2.—Concentrations of FdUMP in the tissues of BDF_1 mice bearing 6-day solid L1210 lymphocytic leukemia after 5-FU-2-C^{14} was administered iv at 200 mg/kg and approximately 300–1000 microcurie/kg

Pooled tissues of 5–10 mice	Concentrations (nmol/g)* of FdUMP at—			
	2 hrs	24 hrs	48 hrs	72 hrs
Tumor	9	83	40	46
Bone marrow	6	5	2	1
Small intestine	13	15	12	16
Kidney	15	16	12	11
Liver	24	15	8	11
Spleen	96	30	79	51
Brain	5	5	2	2

*The concentrations of FdUMP in bone marrow were expressed in nmol/10^9 cells which approximates nmol/g.

animals (at these times) contained only marginally lower concentrations of FdUMP than the liver from tumor-bearing animals, despite evidence for infiltration of tumor cells into the organ (10).

DISCUSSION

The concentrations of radioactivity in murine tissues at 48 and 72 hours after 5-FU-2-C^{14} administration, determined radioautographically and expressed as density, were compared with those determined radiochemically and expressed as nmol/g. The concentrations in the blood (determined by both techniques) were set equal to unity, and the concentrations in the tissues were set relative to these (table 3). The quantitative accuracy of the densitometric method was demonstrated by the relatively good agreement between the results for homogeneous tissues assayed by the two techniques. Determination (using radiochemical techniques) of the concentrations of drug equivalents in rodent blood at times when whole-body radioautography is carried out, provides a means of calculating the concentrations of total drug equivalents in the tissues from the radioautograms. Consequently, be-

TABLE 3.—Normalized total radioactivity concentrations in the tissues of BDF_1 mice bearing 6-day solid L1210 lymphocytic leukemia, expressed relative to the concentration in blood and determined by densitometric and radiochemical techniques after 5-FU-2-C^{14} administration (200 mg/kg and 1000 microcurie/kg iv)*

	Relative total radioactivity concentrations at—			
	48 hrs		72 hrs	
Tissue	Densitometric determination	Radiochemical determination	Densitometric determination	Radiochemical determination
Blood	1	1†	1	1‡
Tumor				
Inner	15	50	7	46§
Outer	300		230	
Bone marrow	6	4	12	4
Small intestine				
Lining	51	23	24	18
Contents	11		4	
Kidney				
Cortex	53	22	23	21
Medulla	32		12	
Renal pelvis	14		7	
Liver	47	33	27	28
Spleen				
Red pulp	105	66	100	54
White pulp	24		10	
Brain				
Forebrain	4	5	5	4
Cerebellum	8		8	

*At various times after injection some mice were frozen and processed for whole-body radioautography, and tissues were removed from others.

†1 = 3.0 nmol/g.

‡1 = 2.4 nmol/g.

§Calculation based upon the concentration of acid-soluble 5-FU equivalents in tumor at 48 hrs.

fore a full-scale preclinical disposition study is undertaken, the radioautographic technique can provide rapid preliminary quantitative data on the distribution of total drug equivalents, besides providing a guide for further biochemical studies. It is also clear that the worth of the densitometric technique lies in the detection and quantitation of drug equivalents in tissues or cells within organs, such as the brain. Such specific loci could not be detected using biochemical techniques.

An obvious disadvantage of the radioautographic technique is that it provides no information on the metabolic composition of the drug equivalents. These equivalents could be composed entirely of nontoxic catabolites. In the present study, most of the radioautographic data were obtained 12 hours or more after 5-FU-2-C^{14} administration. At this time, the majority of the catabolites, such as α-fluoro-β-ureidopropionic acid and CO_2, have been excreted (10,11), indicating that the radioactivity detected represents principally remaining drug and anabolites. For some murine tissues, it has been demonstrated biochemically that this indication is correct. By 24 hours, drug and anabolites including FdUMP made up the majority of the acid-soluble drug equivalents in murine tissues (10), and by 72 hours (in most tissues studied) FdUMP was the metabolite present in the highest concentrations (table 2) (10). Furthermore, comparison of the concentrations of total drug equivalents with the concentrations of FdUMP in the tissues at each time period (tables 1 and 2) demonstrates that, particularly by 72 hours, the distribution of the active metabolite roughly paralleled that of total radioactivity. Therefore, particularly at late time periods, the density of radioactivity determined radioautographically is indicative of the FdUMP concentration.

The above data indicate that for 5-FU a preliminary whole-body radioautographic study would have been a valuable addition to the initial preclinical disposition work. However, for other drugs, such a study would be less meaningful, particularly if the radioactive label and the therapeutic portion of the drug were split by metabolism, as might occur with a nitrosourea or cyclophosphamide.[7]

For any drug studied by the radioautographic technique, it is ultimately necessary (as was done with 5-FU) to determine biochemically the metabolic composition of the total radioactivity in the tissues. However, even superficial knowledge of the metabolism of a drug may aid in the interpretation of whole-body radioautographic data. For example, the active form of pyrimidine and purine analogs such as 5-FU are usually ionized nucleotides formed intracellularly; therefore, radioactivity which persists for hours after administration of such drugs is likely to be of chemotherapeutic importance.

Radioautographic observations correlated with what is known of the rate and routes of excretion of 5-FU and its metabolites by mice after iv administration of 5-FU-2-C^{14} (10); such data are not available for rats. The low concentration of radioactivity in the blood relative to tissues at 2 hours correlates with the rapid disappearance of 5-FU from the blood. Radioactivity was broadly distributed throughout the kidney tissues at 2 hours which is consistent with 60% of the total radioactivity being excreted by the urinary route of which 75% (10) to 100%[8] occurs during the first 2 hours. Radioactivity in the kidney after 24 hours was concentrated principally in the cortex and corticomedullary junction and probably corresponds to drug and metabolite incorporation into kidney tissue or possibly to low concentrations of radioactivity in urine. Another major excretory route for 5-FU is exhalation as CO_2. Thirty-five percent of an intraperitoneal (ip) dose of 5-FU-2-C^{14} given to mice was eliminated by this route (11), taking place rapidly over the first 4 hours and slowly over the next 20 hours. The presence of radioactivity in the parenchyma of the lung and in nasal secretions may be related to $^{14}CO_2$ derived from 5-FU. Radioactivity was observed in the contents of the gallbladder and gastrointestinal tract, including feces, demonstrating the occurrence of biliary secretion and fecal

[7] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan.

[8] Chadwick M. Unpublished data.

excretion. The fecal route of excretion was reported to be negligible in mice (11). The extent of fecal excretion cannot be quantitated from radioautograms. In mice, however, it appeared to be insignificant, whereas in rats it appeared more major. In both species, reabsorption from the intestine of the drug equivalents secreted in the bile may have occurred.

The localization and persistence of 5-FU-derived C^{14} radioactivity was demonstrated in a number of rodent tissues: the mucosal layer of the gastrointestinal tract, the bone marrow, and the cerebellum, which are sites of toxic effects in man. Moreover, FdUMP also persisted in these tissues (table 2).

Gastrointestinal toxic effects, including diarrhea, nausea, emesis, and stomatitis, are common side effects of 5-FU treatment in man (1) and in rodents (9). 5-FU has been shown to cause histopathologic changes in the mucosa of the small intestine (19), colon (20), and rectum (21) in man, and in the gastrointestinal mucosa of rats and mice (22–24). Such changes are consistent with the localization and persistence of radioactivity for 96 hours in the mucosal layer of the gastrointestinal tract of rodents and with the persistence of FdUMP for 72 hours in the small intestine of mice at relatively constant concentrations (table 2).

5-FU causes bone marrow depression in man (1) and rodents (9). At the dose used in the present study, 200 mg/kg administered ip to mice, 5-FU depressed the number of colony-forming units in bone marrow which required 6 days to return to normal (10). This is consistent with the localization and persistence of radioactivity in rodent bone marrow for 96 hours and with the persistence of FdUMP in mouse bone marrow for 72 hours.

After the administration of a drug such as 5-FU, which inhibits nucleic acid synthesis, the presence of radioactivity (particularly after the excretion of catabolites is finished) in cells known to be undergoing division, such as gastrointestinal mucosa and bone marrow, is sufficient to predict toxicity in these cells without analyzing for the active metabolite. However, in cells which are not undergoing division, the presence of radioactivity, even the presence of the active metabolite, is not sufficient to predict for toxicity. A good example in the present study is the concentration of 5-FU equivalents in the liver and the metabolism by the liver of 5-FU to FdUMP without any evident toxicity. However, the concentration of radioactivity in tissues not undergoing cell division can predict for toxicity. Examples in the present study are the cerebellum and the pancreas.

The localization of radioactivity in the Purkinje and granular layers of the cerebellum (fig 2) may be significant because a drug-related acute cerebellar syndrome involving cerebellar ataxia occurred in 3% of patients after systemic 5-FU therapy (7,8). Furthermore, loss of neurons in the granular layer was observed in one patient who died during 5-FU therapy (8). It has been suggested that the neurotoxicity may result from the effects of 5-FU nucleotides on DNA and RNA synthesis (8) or, alternatively, from the inhibition of the Krebs cycle by fluorocitrate produced by the liver and kidney (25,26). FdUMP was detected in the mouse brain at 2, 24, 48, and 72 hours after dosing (10), and, assuming its concentration parallels that of total radioactivity, the nucleotide was present in relatively high concentrations in specific areas of the cerebellum. The importance of FdUMP in causing neurotoxicity is questionable since there is no evidence for DNA turnover in neurons (27). However, RNA turnover does occur (27), and possibly the incorporation of 5-FU into RNA (11,18,28) results in neurotoxicity. Alternatively, if neurotoxicity in man is a result of systemic fluorocitrate production, the localization of radioactivity in the cerebellum of rodents may be coincidental.

Radioactivity was found uniformly distributed throughout the pancreas in relatively high concentrations. At 48 hours, the concentration of radioactivity in the rat and mouse pancreas (determined densitometrically) was 100 times that found in the blood (figs 1 and 3). This distribution may be related to pancreatic toxic effects reported in rats after 5-FU treatment (29). 5-FU resulted in the production of zymogen granules with reduced lipase activity and blocked the release of the granules from the cell. Pancreatic toxic effects have not been reported after 5-FU therapy in man. However, drug-induced changes in

the structure and function of the human pancreas could possibly contribute to gastrointestinal toxic effects observed in man, particularly in view of the reported lack of correlation between the development of diarrhea and histopathologic changes in the gastrointestinal mucosa (19–21).

The cortex of the solid L1210 tumor contained some of the highest concentrations of radioactivity which persisted for over 96 hours. The unequal distribution of radioactivity between the inner and outer layers of the tumor mass was probably a result of poor vascularization at the center of the advanced tumor. FdUMP was also found at high concentrations in the tumor, persisting for 72 hours (table 2). It is significant that the dose of 5-FU used in this study was shown to be therapeutically effective, resulting in a 50% decrease in size of the local tumor (10).

The persistence of radioactivity in the small intestine, bone marrow, and tumor for 96 hours after dosing, together with the persistence of the postulated active metabolite, correlates with the clinical finding that 5-FU administered by single weekly injection (15 mg/kg) is less toxic to normal tissues and equally effective against colorectal and other tumors as is 5-FU administered in monthly courses (15 mg/kg/day for 5 days, followed by 7.5 mg/kg every other day until mild toxicity ensues) (30,31). Administering the drug at weekly intervals presumably allows time for the concentration of the active metabolite in normal tissues to decline so that they recover from the toxic effects before the next dose is administered. In the tumors, some of which are slower growing than the corresponding normal tissues (32), the active metabolite must persist at a concentration sufficient for oncolytic action. Radioautographic studies, therefore, can provide a guide for determining the most suitable dose schedule for man. Rapid disappearance of drug-derived radioactivity from the tissues would indicate that the drug should be administered at frequent intervals. Persistence of radioactivity, particularly after a drug such as 5-FU for which the active metabolite is a polar intracellular nucleotide, would indicate that administration of the drug at infrequent intervals would be the most successful schedule, particularly against slow-growing tumors.

Often, full-scale physiologic disposition experiments using biochemical techniques are carried out after the drug has entered the clinic. This retrospective study on the physiologic disposition of 5-FU in rodents demonstrates that radioautographic studies can provide rapid preliminary quantitative data on the distribution of total drug equivalents in rodent tissues. Such data serve as a guide and adjunct for future biochemical studies. Also, if these data are combined with preliminary biochemical data, such as blood levels of the parent compound and urinary and fecal excretion of total drug equivalents, they may serve as a useful model for predicting drug toxicity and suitable therapy regimens in man.

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Effectiveness of the Nitrosoureas as a Function of Their Lipid Solubility in the Chemotherapy of Experimental Rat Brain Tumors [1,2,3]

Victor A. Levin and Pokar Kabra [4,5,6]

SUMMARY

The increase in lifespan for Fischer 344 rats intracerebrally inoculated with cultured cells from a 1-methyl-1-nitrosourea-induced sarcoma was compared for six nitrosoureas of log P (1-octanol/water partition coefficient) from -0.41 to 3.3. Each drug was given at different doses (μmol/kg basis) and their effectiveness was compared and contrasted to the log P for each drug. The relationship of log 1/C to log P was best fitted by a linear function from log P 0.37 to 3.3. Optimum log P was 0.37.

[Cancer Chemother Rep 58:787–792, 1974]

A critical consideration in the chemotherapy of brain tumors is attaining an effective drug concentration in the areas immediately surrounding the main tumor. This objective assumes importance because most brain tumors are infiltrative in character and malignant cells infiltrate areas of normal brain in advance of tumor-induced neovascularization. Unlike the main tumor, these regions have permeability characteristics determined by normal or nearly normal capillaries (1,2). In this situation, lipophilic drugs are more effective than water-soluble drugs with respect to drug concentration attained in the brain immediately adjacent to the tumor (2–5).

Present clinical experience supports the above theory in that currently, all effective agents are lipid soluble. Two of the most widely used and effective agents are the nitrosoureas, BCNU and CCNU; both have shown antitumor effects in human and animal brain tumors (5–12). Procarbazine, a methyl hydrazine analog which is rapidly oxidized to a lipophilic azo derivative in the brain,[7] has recently shown efficacy against human malignant brain tumors comparable to the nitrosoureas (13).

Impetus for the following study came from (*a*) our desire to determine the optimum lipid solubility characteristics of chemotherapeutic agents effective against brain tumors, (*b*)

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[2] Supported in part by Public Health Service grant CA-15435 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare; the Joe Gheen Medical Foundation; and the Association for Brain Tumor Research.

[3] Compounds used in this study were supplied by Drug Research and Development, Division of Cancer Treatment, NCI. Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

[4] Departments of Neurological Surgery (H. C. Naffziger Laboratories for Neurosurgical Research; Drs. Levin and Kabra), Neurology (School of Medicine; Dr. Levin), and Pharmaceutical Chemistry (School of Pharmacy; Dr. Levin), University of California, San Francisco.

[5] We would like to thank Drs. Charles Wilson, Corwin Hansch, and Glynn Wheeler for encouragement and constructive criticism of this study, and Dr. Wheeler for determining the physiochemical and biochemical parameters on PCNU and NSC-105763. The technical assistance of Carol Maroten and Mary Freeman is greatly appreciated.

[6] *Reprint requests to:* Victor A. Levin, MD, Department of Neurological Surgery, University of California School of Medicine, San Francisco, Calif 94143.

[7] Kabra PM, Levin VA, and Weinkam R. Unpublished observations.

clinical and experimental animal studies indicating superiority of BCNU over CCNU, and (*c*) the work of Hansch and others who suggested that within a group of lipid-soluble drugs lipid solubility both above and below an optimal range decreases chemotherapeutic effectiveness (14–16). Since lipid solubility is one critical factor in the chemotherapy of brain tumors, we compared the antitumor effectiveness of six nitrosourea compounds of widely divergent lipid solubility in a transplantable intracerebral (ic) rat tumor (9L).

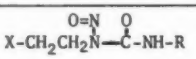
MATERIALS AND METHODS

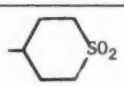
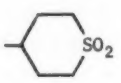
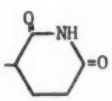
The tumor used in these studies was originally induced with 1-methyl-1-nitrosourea and has been carried by serial subcultures and animal passage as 9L sarcoma (17,18). To produce the ic tumors, a suspension of 4×10^4 cells in 0.01 ml of balanced salt solution was injected stereotactically into the centrum ovale of the parietal lobe of male Fischer 344 rats. Animals were maintained in their cages on Purina rat chow and water ad libitum.

The drugs, CCNU, methyl-CCNU, PCNU, NSC-105763, NSC-106767, and BCNU, were refrigerated at −60°C in their lyophilized form until used. Drugs were weighed immediately prior to use and suspended in 0.3% hydroxypropyl cellulose (Klucel). Each animal was injected intraperitoneally (ip) with 0.1 ml/100 g of body weight. Drugs were injected into all animals on the 14th day after implantation.

The drug doses shown in table 1 were chosen to include the optimum dose for each of the six nitrosoureas.

TABLE 1.—Nitrosourea treatment of ic rat sarcoma 9L on Day 14 after tumor implantation: relationship of dose to log P



Drug	X=	R=	Log P	Dose (μmol/kg)	RLS (%)	P*
NSC-106767	F		−0.41	11	106	NS
				22	117	<0.0001
				37	78	NS
				56	109	NS
NSC-105763	Cl		0.19	14	110	NS
				28	121	<0.0001
				35	133	<0.0001
				53	136	<0.0001
PCNU	Cl		0.37	32	158	<0.0001
				40	165	<0.0001
				95	189	<0.0001
				183	107	NS
BCNU	Cl	CH_2CH_2Cl	1.53	47	122	<0.0001
				59	132	<0.0001
				67	160	<0.0001
				93	136	<0.0001
CCNU	Cl		2.83	61	119	<0.0001
				86	148	<0.0001
				94	152	<0.0001
				128	125	<0.0001
Methyl-CCNU	Cl		3.3	120	115	<0.0001
				136	139	<0.0001
				152	136	<0.0001
				180	118	<0.0001

*Determined using the Wilcoxon rank sum analysis modeled after Gehan (21). NS = not significantly different from control.

All rats were checked daily. Random animals were autopsied at death and the brains were removed and examined for tumor location and size.

The data were analyzed for median survival time and RLS. The latter was defined:

$$\text{RLS} = \frac{\text{median survival of treated animals}}{\text{median survival of control animals}} \times 100. \quad (1)$$

RESULTS

Three hundred animals were used in these studies. The percent tumor take, based on a lack of control survivors after 37 days, was 100%. In the six control groups the median survival of ic 9L controls was 25.8±2.2 (SE) days.

Table 1 summarizes the RLS attained for each drug at the various doses used. It is apparent that NSC-106767 and NSC-105763 are relatively ineffective against ic 9L whereas the more lipophilic drugs, beginning with PCNU as listed in the table, show significant antitumor activity.

The drugs are further compared in table 2 where log 1/C has been determined for each drug when possible. C is defined here as the moles of drug/kg of body weight which would result in an RLS of 130, 140, 150, and 160 respectively. It is apparent that PCNU is the most effective drug of the six tested. When log P (1-octanol/water partition coefficient) from 0.37 to 3.3 was compared to log 1/C at the various RLS values above, the relationship was found to be linear. Table 3 summarizes the slopes and intercepts determined by a linear regression analysis of the data. The data could not be fitted to a parabola as is obvious from the plot of the actual data in figure 1.

Averaging the slopes and intercepts from RLS values 130–160 a general relationship of log P to log 1/C was found to be:

$$\text{Log } 1/C = -0.282 \log P + 4.745. \quad (2)$$

Using the equation above it was calculated that PCNU was the most effective drug and that BCNU was 48%, CCNU was 21%, and methyl-CCNU was 15% as effective as PCNU.

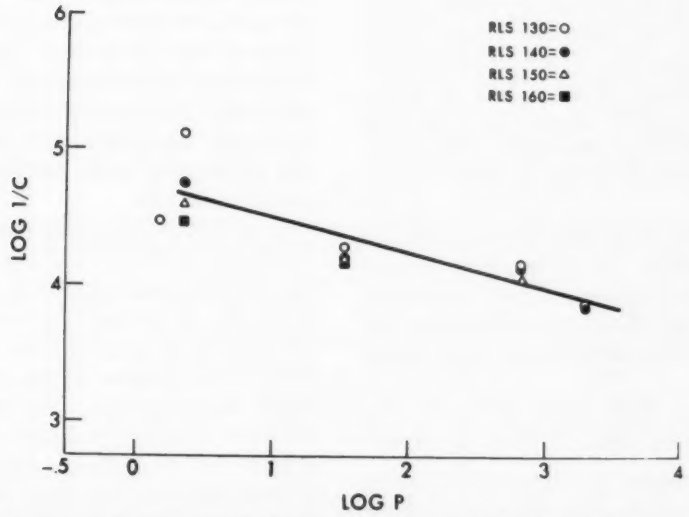


FIGURE 1.—Least squares linear regression analysis of log P versus log 1/C.

TABLE 2.—Antitumor activity of nitrosoureas against ic rat sarcoma 9L: relationship of log P to log 1/C

Drug	Log P	Log 1/C for RLS—			
		130	140	150	160
NSC-105763	0.19	4.45	4.41*	—	—
PCNU	0.37	5.10	4.74	4.59	4.46
BCNU	1.53	4.28	4.24	4.18	4.17
CCNU	2.83	4.17	4.12	4.05	—
Methyl-CCNU	3.30	3.88	3.82	—	—

*Value was extrapolated even though upper-limit of RLS was not achieved; this value is a maximum and most likely log 1/C is lower.

TABLE 3.—Least squares linear regression analysis: log P vs log 1/C

RLS	Slope	Intercept	SE*	R†	N‡
130	–0.3833	5.114	0.16	0.94	4
140	–0.2778	4.788	0.09	0.96	4
150	–0.2171	4.616	0.07	0.95	3
160	–0.2500	4.460	—	—	2
130–160	–0.2643	4.754	0.17	0.86	13
Mean for 130–160:	–0.2821	4.745			

*SE = standard error of the estimate.
†R = correlation coefficient.
‡N = number of values used in regression analysis.

DISCUSSION

In the present study, animals were treated relatively late after tumor implantation, at a time when the tumors contained approximately 6×10^7 cells (60 mg) (18).[8] This time period was chosen to approximate the time at which a typical patient might be given brain tumor chemotherapy (eg, symptomatic large tumor with low growth fraction). In this tumor system, the best response (RLS) on Day 14 to any single chemotherapeutic agent to date has been no more than 160.[9] Therefore, table 1 reflects a rather narrow range of RLS values for each drug.

For the most part, each drug has a parabolic dose-RLS relationship. At a low drug concentration a low RLS reflects poor antitumor activity and at a high drug concentration a low RLS reflects toxicity, particularly liver toxicity.

Using 1/C for RLS (130–160), it was clearly demonstrated that PCNU, a piperidine-2,6-dione, chloroethyl-nitrosourea, was the most effective drug tested. Further, within a log P range of 0.37–3.3, drug activity was linearly related to log P. The fact that the data are related in a linear rather than a parabolic manner may reflect a lack of sufficiently active drugs below log P 0.37. The activity at log P 0.37 was somewhat surprising in view of the fact that PCNU is 20%–25% ionized at physiologic pH (16) and that the optimum therapeutic effect and presumably optimum solubility for blood-brain passage for hypnotics and barbiturates is approximately 2.0 (14).

It is interesting that Hansch et al found an optimum log P = –0.6 (eg, water soluble) for the nitrosoureas against ic and systemic L1210 (16). In all probability however, ic L1210 is not typical of ic neoplasms since it involves an infiltrative process of the meninges and is not an ic tumor per se. The increase in lifespan of mice bearing ic L1210 may also be a reflection of its systemic activity on circulating L1210 cells rather than its central nervous system activity (16). The fact that only lipophilic drugs have shown efficacy against

[8] Hoshino T, and Barker M. Unpublished observations, July 1972.

[9] Levin VA, and Barker M. Unpublished observations, July 1973.

brain tumors is further evidence that the findings in ic L1210 are not extrapolatable to solid ic tumors. In another study, Montgomery et al used the Lewis lung tumor model and compared the effects of 13 nitrosoureas (19). It was found that the relationship of log P to log 1/C, with few exceptions, was parabolic with an optimum log P = 0.8. Those results are more consistent with our present data than the meningeal L1210 studies.

To explain the dependence of antitumor activity in L1210 to log P, Hansch et al proposed that highly lipophilic drugs would bind to serum proteins more so than the less lipophilic ones and hence reduce the plasma pool of free drug (16). In essence, bound drug could undergo decomposition while bound, thus reducing the probability of reaching the active site (eg,tumor DNA).

To an extent, however, instability in plasma would be detrimental to solid tumor chemotherapy of the brain because the reactive moieties would combine in the plasma and extracellular fluids reducing their effective intracellular and intranuclear concentrations unless these drugs were less stable in the intracellular rather than the extracellular environment.

Wheeler et al recently compared the chemotherapeutic activity of 17 nitrosoureas against ip L1210 to in vitro alkylating and carbamoylating activity and found that, in general, alkylating activity was directly related to antitumor activity and increased with decreasing lipophilicity (20). Carbamoylating activity was found to be inversely related to antitumor activity and correlated more closely to systemic toxicity. Some of their data are found in table 4 together with the relative antitumor activity of the nitrosoureas in our ic tumor model. Dr. Wheeler kindly determined the relative alkylating activity of PCNU and NSC-105763, the carbamoylating activity of PCNU, and the half-lives of the two drugs in ethanol/phosphate buffer ($\frac{1}{19}$) to complete our table.

Clearly, our data support the concepts of Wheeler et al (20) in terms of the relationship of antitumor activity to alkylating activity and those of Montgomery et al (19) in terms of optimum log P for solid tumor chemotherapy.

The implications of these studies are that for the nitrosoureas, a minimum lipophilicity of approximately log P = 0.4 is required and desired for effective chemotherapy of solid tumors within the brain. The fact that this differs little from what is necessary for effective chemotherapy in another solid tumor, Lewis lung (eg, log P = 0.8), but is substantially less than for the optimum activity of barbiturates (eg, log P = 2), suggests that a strict reliance on log P for proposed brain tumor agents may be premature since other factors such as mode of drug action, rates of intracellular biotransformation, binding constants, and inherent tumor sensitivity must be determined and evaluated together with parameters such as log P to understand the totality of what constitutes effective solid brain tumor chemotherapy.

Lastly, based on the superior antitumor activity of PCNU compared to BCNU, CCNU, and methyl-CCNU, its greater alkylating activity, and its low carbamoylating activity,

TABLE 4.—Relationship of antitumor activity, log P, drug half-life, alkylating activity, and carbamoylating activity (all comparisons are relative to PCNU which has been taken as 100%)

Drug	Log P	Antitumor activity	$T_{0.5}$*	Alkylating activity*	Carbamoylating activity*
NSC-106767	−0.41	—	72	162	108
NSC-105763	0.19	—	85†	128†	—
PCNU	0.37	100	100†	100†	100†
BCNU	1.53	48	163	75	285
CCNU	2.83	21	201	28	417
Methyl-CCNU	3.30	15	201	28	379

*Half-life, $T_{0.5}$ (ethanol/phosphate buffer), relative alkylating activity, and relative carbamoylating activity from Wheeler et al (20).

†Kindly determined by Dr. G. P. Wheeler, Southern Research Institute, Birmingham, Ala.

PCNU should be evaluated further as a potentially useful brain tumor chemotherapeutic agent. If the relationship of alkylation to antitumor activity and carbamoylation to toxicity is valid, it would be expected that PCNU would have a much greater margin of safety than BCNU, CCNU, or methyl-CCNU.

LIST OF COMPOUNDS

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-

1-Methyl-1-nitrosourea: NSC-23909; CAS reg. No. 684-93-5

NSC-105763: CAS reg. No. 33022-02-5; urea, 1-(2-chloroethyl)-1-nitroso-3-(tetrahydrothiopyran-4-yl)-, *S,S*-dioxide

NSC-106767: CAS reg. No. 32319-89-4; urea, 1-(2-fluoroethyl)-1-nitroso-3-(tetrahydrothiopyran-4-yl)-, *S,S*-dioxide

PCNU: NSC-95466; CAS reg. No. 13909-02-9; urea, 1-(2-chloroethyl)-3-(2,6-dioxo-3-piperidyl)-1-nitroso-

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-*α*-(2-methylhydrazino)-, monohydrochloride

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Inhibition of L-Asparagine Synthetase by a New Amino Acid Antibiotic With Antitumor Activity: L-(αS,5S)-α-Amino-3-chloro-4,5-dihydro-5-isoxazoleacetic Acid (NSC-163501) [1,2,3]

David A. Cooney,[4] Hiremagalur N. Jayaram,[4] Joan A. Ryan,[5] and Vincent H. Bono [4,6,7]

SUMMARY

NSC-163501 and its hydroxy analog (αS,4S,5R)-α-amino-3-chloro-4-hydroxy-4,5-dihydro-5-isoxazoleacetic acid (NSC-176324) are powerful inhibitors of the utilization of L-glutamine by L-asparagine synthetase both in vitro and in vivo. The type of inhibition produced in vitro is concluded to be apparently competitive on the basis of graphic analysis. L-Glutamine in large molar excess effectively antagonizes inhibition produced by these agents; however, established inhibition is concluded to be irreversible on the basis of dialysis experiments. Attempts to demonstrate alteration of the inhibitor molecule in the act of catalysis were unsuccessful.

[Cancer Chemother Rep 58:793–802, 1974]

NSC-163501 (fig 1) was recently isolated and characterized by Hanka and his co-workers as an amino acid antibiotic with significant antitumor activity against L1210 leukemia (1,2). On structural grounds, Dr. M. Rabinovitz of the National Cancer Institute proposed that the compound might be an analog of L-glutamine. Since our laboratory has been engaged for some time in a systematic search for inhibitors of L-asparagine synthetase, an enzyme catalyzing amide donation by L-glutamine to L-aspartic acid (3), we examined the ability of this new antibiotic to inhibit the synthesis of L-asparagine in extracts of L5178Y leukemia rendered resistant to L-asparaginase by subcurative doses of the enzyme. In addition, inhibition was tested against crude L-asparagine synthetase of murine pancreas, in light of recent observations which established that organ as the site of the most active synthesis of L-asparagine in mammals (4).

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[2] Supported in part by contract N01-CM-33728 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] NSC-163501 and NSC-176324 were supplied by The Upjohn Company, Kalamazoo, Mich. All chemicals not specifically identified by supplier in the text were of reagent grade. Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

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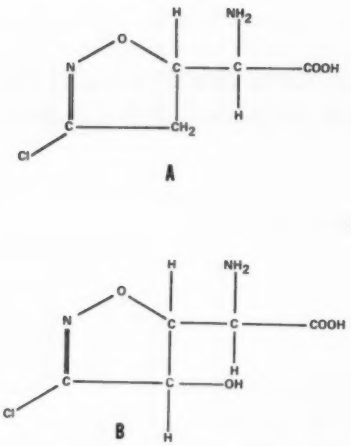

FIGURE 1.—Structures of NSC-163501 (*A*) and NSC-176324 (*B*).

MATERIALS AND METHODS

Preparation of Partially Purified Soluble L-Asparagine Synthetase

Tumors were collected from male BDF_1 mice 7–10 days after subcutaneous (sc) injection of 1×10^5 L5178Y leukemia cells rendered resistant to L-asparaginase (L5178Y/AR). The tumors were homogenized in 9 volumes of 0.1 M tris-HCl buffer (pH 7.6) containing 0.5 mM EDTA and 1 mM dithiothreitol. The homogenate was centrifuged in the cold at 100,000 *g* in a Beckman L2-65B ultracentrifuge for 30 minutes and the supernatant (specific activity, 0.001 IU/mg of protein) was collected. A portion of this supernatant was used as a source of crude L-asparagine synthetase. The remainder was partially purified by heating at 50°C for 10 minutes after treating with adenosine-5′-triphosphate disodium salt (ATP),[8] $MgCl_2$, L-glutamine, ammonium chloride, and dithiothreitol, each at a concentration of 0.01 M. The heated extract was immediately chilled on ice and centrifuged to remove the precipitated proteins. The supernatant was brought to 30% saturation with ammonium sulfate and centrifuged at 18,000 *g* for 30 minutes. The precipitate was discarded and the supernatant brought to 50% saturation with ammonium sulfate. After centrifugation, the precipitate was suspended in 0.005 M tris-HCl buffer (pH 7.4), containing 0.001 M dithiothreitol and 10% glycerol, and then dialyzed against three changes of the same buffer for 3 hours. This dialyzed extract (specific activity, 0.0058 IU/mg of protein) was used as the source of partially purified L-asparagine synthetase of tumoral origin for the in vitro inhibition studies.

For the preparation of L-asparagine synthetase of normal pancreas, normal BDF_1 male mice weighing between 18 and 20 g were killed, and their pancreata collected and homogenized in 9 volumes of 0.1 M tris-HCl buffer (pH 7.6) containing 0.5 mM EDTA and 1 mM dithiothreitol. The homogenate was centrifuged in the cold at 12,000 *g* for 20 minutes. As before, a portion of this supernatant was used as the source of crude pancreatic L-asparagine synthetase. The remainder was partially purified as above. The resultant dialyzed enzyme (specific activity, 0.001 IU/mg of protein) was used as the source of pancreatic L-asparagine synthetase for inhibition studies carried out in vitro.

Measurement of L-Asparagine Synthetase

L-Asparagine synthetase (EC 6.3.5.10) was measured by a modification of the enzymatic radiometric technique of Cooney et al (5); the theory and full details will be published elsewhere. Briefly, in a final volume of 10 μl the following were admixed: 2.5 μmol of tris-HCl buffer (pH 8.4), 0.2 μmol of ATP-$MgCl_2$, 0.1 μmol of L-glutamine or 0.5 μmol of ammonium chloride, 0.125 microcuries of L-[4-^{14}C]aspartic acid[9] (specific radioactivity, 22.2 microcuries/μmol), and 5 μl of crude or partially purified L-asparagine synthetase from tumor or pancreas. After 10–30 minutes of incubation at 37° C the vessels were heated at 95° C for 5 minutes. Any residual L-aspartic acid was then β-decarboxylated by the addition to each vessel of 50 μl of a decarboxylation reagent consisting of 6.8 mM α-ketoglutaric acid[10] and 8.5 mM zinc sulfate in 0.66 M sodium acetate buffer (pH 5.0) containing 0.2 mg/ml of L-glutamate

[8] Obtained from Sigma Chemical Co., St. Louis, Mo.

[9] Purchased from the Biochemical Nuclear Corp., Burbank, Calif.

[10] A product of Calbiochem, Los Angeles, Calif.

oxaloacetate transaminase.[11] The open vessels were incubated at 37° C for 4 hours, whereafter 2 IU of L-asparaginase from *Escherichia coli* was added. At the same time a droplet of a saturated solution of barium hydroxide was deposited on the underside of each vessel's lid to trap $[^{14}C]O_2$ arising from the zinc-catalyzed β-decarboxylation of oxaloacetic acid, arising in turn from the transamination with α-ketoglutaric acid of any L-aspartic acid produced by the amidohydrolysis of L-asparagine. When inhibitor constants were investigated, sufficient drug was lyophilized on the bottom of the plastic reaction vessel, so that on reconstitution with the 10-μl final reaction volume, the intended molarities were achieved. This procedure led to no measurable alteration of the absorption spectrum of the inhibitor or of the potency of inhibition.

Attempts to Counteract Inhibition

Attempts were made to determine if the ordinary substrates of L-asparagine synthetase could prevent or reverse the inhibition produced by NSC-163501. For this purpose, the drug was added to 50 μl of enzyme solution to a final molarity of 1.0 mM either 10 minutes before or after the addition of L-glutamine (10 and 100 mM), ammonium chloride (10 and 100 mM), ATP-$MgCl_2$ (10 mM), L-aspartic acid (0.5 mM), or dithiothreitol (0.5–10 mM). After 30 minutes at 37° C, the enzyme was diluted 4–20-fold with 0.1 M tris-HCl buffer (pH 7.6) and assayed in the usual manner. Overnight dialysis was carried out at 4° C in cellulose dialysis tubes [12] against three changes of 1 liter each of 0.1 M tris-HCl buffer (pH 7.6) containing 0.5 mM EDTA and, in some cases, 1 mM dithiothreitol.

Preparation of Irreversibly Inhibited Enzyme

L-Asparagine synthetase was partially purified by ammonium sulfate fractionation, as described earlier, and subjected to DEAE-Sephadex (A-50) chromatography to a specific activity of 0.03 IU/mg of protein. The enzyme solution was rendered 0.01 M in tris-HCl buffer (pH 8.4) or 1 mM in NSC-163501 or 2 mM in neutralized mucochloric acid, incubated at 37° C for 30 minutes, and then dialyzed against a large excess of 0.01 M tris-HCl buffer (pH 8.4) containing 20% glycerin (vol/vol), 1 mM dithiothreitol, and 0.5 mM EDTA.

In Vivo Studies

For the in vivo studies of the effect of NSC-163501 on tumoral and pancreatic L-asparagine synthetase, male BDF_1 mice weighing between 18 and 20 g were injected by the sc route with 10^5 cells of L5178Y/AR leukemia. Seven days later, the mice were injected intraperitoneally (ip) with the specified dose of the inhibitor. Four hours later the animals were killed, and the tumors and pancreata were collected and homogenized in 0.1 M tris-HCl buffer (pH 7.6) containing 0.5 mM EDTA and 1 mM dithiothreitol. The homogenate was centrifuged at 18,000 *g* for 5 minutes before the measurement of L-asparagine synthetase activity.

Binding Experiments

To 1-ml aliquots of partially purified dialyzed L-asparagine synthetase was added either 50 μmol of tris-HCl buffer (pH 7.4) or 2 μmol of NSC-163501. After 15 minutes at 25°C the reaction mixtures were transferred to Centriflo cones (Amicon Corp., Lexington, Mass) and centrifuged at 1500 *g* for 2 hours to separate unbound from bound drug. The diafiltrate was collected and subjected to automatic amino acid analysis (6). Using 0.25 M lithium citrate buffer (pH 7.2), NSC-163501 elutes from the long column of the amino acid analyzer at a position midway between L-aspartate and L-threonine. To the retentate was added 1 ml of 1 N HCl and the resultant assemblies were centrifuged at 1500 *g* at 4° C for 2 hours. The filtrates were collected and analyzed as described. One ml of 1 N NaOH was added to the acid retentate. After 2 hours at 4° C the assemblies were processed as described earlier.

RESULTS AND DISCUSSION

Both NSC-163501 and its hydroxy analog (NSC-176324) are potent in vitro inhibitors of L-asparagine synthetase from mouse tumor and pancreas (table 1). Only the utilization of L-glutamine is interrupted (fig 2); that is to

[11] A product of Boehringer, New York, NY.

[12] Obtained from Union Carbide Corp., Chicago, Ill.

TABLE 1.—In vitro inhibition of L-asparagine synthetase from L5178Y/AR tumor and normal mouse pancreas by NSC-163501 and NSC-176324*

Inhibitor	Concentration (mM)	Inhibition of L-asparagine synthetase (%)			
		L5178Y/AR leukemia: substrates		Mouse pancreas: substrates	
		L-Glutamine	Ammonia	L-Glutamine	Ammonia
NSC-163501	1.0	85	0	90	0
	0.1	52	0	61	0
	0.01	8	0	0	0
NSC-176324	1.0	79	0	85	0
	0.1	23	0	29	0
	0.01	8	0	0	0
NSC-124412 (CONV)	1.0	80	0	91	43
	0.1	66	0	79	39
	0.01	0	0	0	0

*Ten μl of inhibitor, at the molarities specified, was lyophilized in the bottom of an Eppendorf conical reaction vessel. Subsequent measurements of L-asparagine synthetase were carried out as described in the Materials and Methods section in a final volume of 10 μl using partially purified L-asparagine synthetase from L5178Y/AR leukemia or from the pancreas of normal BDF_1 mice.

say, the enzyme can still synthesize L-asparagine at a normal rate with ammonia as a precursor of the amide, even after its utilization of L-glutamine has been inhibited. This finding is analogous to that seen when 5-chloro-4-oxo-L-norvaline (NSC-124412; CAS reg. No. 15148-06-8) inhibits certain amide transferases capable of using either L-glutamine or ammonia (7).[13] These isoxazoles are clearly behaving as selective antagonists of L-glutamine.

Like many other amide transferases, L-asparagine synthetase from L5178Y/AR leukemia can function as an L-glutaminase even in the absence of its other substrates. A partially purified preparation of this enzyme (exposed to NSC-163501 for 30 minutes at a final concentration of 1.0 mM and then exhaustively dialyzed against 0.01 M tris-HCl buffer [pH 7.4] containing 1.0 mM dithiothreitol and 0.5 mM EDTA) retained only 2% of the L-glutaminase activity of an uninhibited control sample.

It therefore became of interest to determine whether L-glutamine (and ammonium chloride,

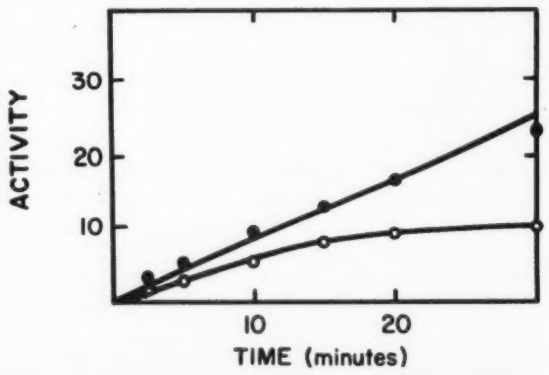

FIGURE 2.—Time-course of selective inhibition of L-glutamine utilization by L-asparagine synthetase incubated with NSC-163501. Inhibitor was dissolved in water, neutralized, and lyophilized at the bottom of Eppendorf 1500-μl centrifuge vessels, so that on reconstitution the final concentration of NSC-163501 was 1 mM. Measurement of L-asparagine synthetase was carried out as described in the Materials and Methods section. The reaction was terminated with heat (95° C, 5 min) at the times indicated. L-Glutamine utilization is indicated with open circles (○) and ammonia utilization with closed circles (●). L-Asparagine synthetase activity is expressed in nanomoles of L-asparagine formed per milligram of protein.

[13] NSC-124412 does inhibit both L-glutamine and ammonia utilization by L-asparagine synthetase of RADA1 leukemia, although inhibition is considerably stronger when L-glutamine is the amide donor (11).

L-aspartate, ATP-$MgCl_2$, or dithiothreitol) could prevent or reverse the inhibition reported above. For this purpose, tumoral synthetase was exposed to NSC-163501 at a concentration of 1.0 mM, diluted, and assayed. Of the several substrates examined, L-glutamine alone protected the enzyme from inhibition; only when the amide was present in 100-fold molar excess over the isoxazole could a protective effect be demonstrated. Even in this case, protection was subtotal (table 2). It is noteworthy that dithiothreitol at a concentration of 10 mM was also effective as a counteragent.

In figure 3, the time-course of the inhibition of L-asparagine synthetase from L5178Y/AR leukemia by NSC-163501 is presented graphically. It can be seen that the rate of interaction is initially rapid, so that approximately 50% of the inhibition ultimately effected is produced within 2–5 minutes of exposure to the agent. Also evident in the figure is the partial protective action of L-glutamine at a concentration of 0.1 M and the efficacious protective action of dithiothreitol at a concentration of 10 mM.

TABLE 2.—Effect of antagonists on the inhibition of L-asparagine synthetase by NSC-163501*

Antagonist	Concentration (mM)	Prevention of inhibition (%)
L-Glutamine	10.0	0
	100.0	31
NH_4Cl	10.0	7†
	100.0	21†
L-Aspartate	0.5	4
ATP-Mg^{++}	10.0	0
Dithiothreitol	0.5	4
	1.0	12
	5.0	33
	10.0	100

*Drug was added to 50-μl aliquots of partially purified preparation of L-asparagine synthetase from L5178Y/AR leukemia to a final molarity of 10 mM either 10 minutes before or after the antagonists. After 30 minutes at 37°C, the enzyme was then diluted 1:4–1:20 with ice-cold 0.1 M tris-HCl (pH 7.6) containing 0.5 mM EDTA and assayed immediately. Measurements of L-asparagine synthetase were conducted according to the methodology presented in the Materials and Methods section.

†This prevention of inhibition is probably more apparent than real, ie, ammonium chloride carried over into the final reaction vessels along with the treated enzyme permitted some synthesis of L-asparagine in its capacity as an alternate substrate whose utilization is not inhibited by NSC-163501.

Graphic analysis of the types and constants of the inhibition produced by NSC–163501 showed that the antibiotic appeared to behave as a competitive inhibitor against L-glutamine (fig 4) but as a noncompetitive inhibitor against L-aspartic acid (not shown). With L-glutamine as substrate, a K_i of 4×10^{-5} M was measured.

Other analogs of L-glutamine appear to interact with amidohydrolases or amide transferases in a strictly competitive way but are in fact irreversible inhibitors (8,9); this feature, in a sense, vitiates the interpretation of the kinetic patterns of inhibition (10). We therefore elected to examine the reversibility of the inhibition exerted by NSC–163501. For this purpose, a preparation of partially purified tumoral synthetase was exposed to a concentration of NSC–163501 calculated to achieve about 75% inhibition and was then dialyzed to remove unbound low molecular weight materials, as described in the Materials and Methods section. Untreated enzyme, carried through the dialysis step, served as control. Analysis of the dialyzed preparation showed that inhibition by NSC–163501 was essentially irreversible; thus, there was a 92%–94% inhibition of L-glutamine utilization by L-asparagine synthetase both before and after exhaustive dialysis, and the presence of 1 mM dithiothreitol in the outer bath failed to influence this inhibition. That the dialyzed enzyme had maintained its integrity was revealed by the finding that its utilization of ammonia (at saturating concentrations) for the synthesis of L-asparagine was equivalent to that of the uninhibited preparation.

When the kinetic behavior of such an irreversibly inhibited, dialyzed enzyme (hereinafter referred to as the "isoxazole enzyme") was compared with that of the uninhibited dialyzed species, it was found that the K_m for L-glutamine of surviving synthetase was unaltered while the V_{max} was reduced tenfold. On the other hand, the V_{max} of the surviving synthetase, with ammonia as substrate, was unaltered, whereas the K_m of the enzyme for

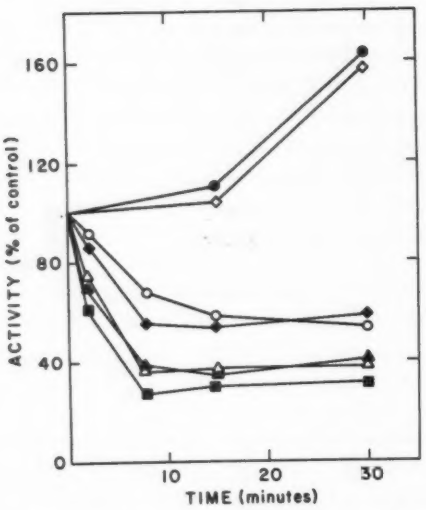

FIGURE 3.—Time-course of the inhibition of L-asparagine synthetase from L5178Y/AR leukemia by NSC-163501; the influence of counteragents. One hundred μl of an extract L5178Y/AR leukemia (30%–50% ammonium sulfate fraction, dialyzed for 8 hrs against 0.1 M tris-HCl buffer [pH 7.6] containing 0.5 mM EDTA and 20% glycerin) was added to 9 Eppendorf 1500-μl conical vessels containing, in a final volume of 125 μl, either 0.125 μmol of inhibitor (△), 0.125 μmol of inhibitor plus 8 μmol of L-glutamine (○), 0.125 μmol of inhibitor plus 0.125 μmol of L-aspartic acid (▲), 0.125 μmol of inhibitor plus 1.25 μmol of ATP-$MgCl_2$ (■), 0.125 μmol of inhibitor plus 8 μmol of L-glutamine plus 0.125 μmol of L-aspartic acid plus 1.25 μmol of ATP-$MgCl_2$ (◆), 0.125 μmol of inhibitor plus 1.25 μmol of dithiothreitol (●), or 1.25 μmol of dithiothreitol without inhibitor (◇). At the times indicated, 20 μl of the incubation mixture was removed and added to 480 μl of ice-cold 0.1 M tris-HCl buffer (pH 7.6) containing 0.5 mM EDTA. The vessel was inverted and 5 μl of the resulting mixture was assayed for L-asparagine synthetase as described in the Materials and Methods section.

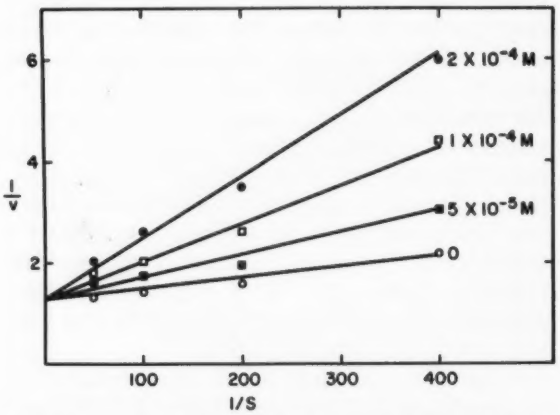

FIGURE 4.—Double-reciprocal plot of the inhibition of L-glutamine utilization by L-asparagine synthetase from L5178Y/AR leukemia by NSC-163501. Inhibitor was dissolved in 0.05 M tris-HCl buffer (pH 8.4); a 10-μl aliquot of the requisite molarity (2×10^{-4} M, 1×10^{-4} M, 5×10^{-5} M) was dispensed in the bottom of Eppendorf 1500-μl vessels and lyophilized. At the time of the assay, the lyophilized inhibitor dissolved immediately in the reaction mixture. The substrate concentrations were: L-glutamine (0.02 M), $MgCl_2$ (0.01 M), ATP (0.01 M), L-aspartic acid (0.56 mM), and tris-HCl buffer (0.25 M). Incubations were done for 10 min at 37°C. Subsequent steps in the measurement of L-asparagine synthetase were carried out as described in the Materials and Methods section. 1/v represents the reciprocal of the nanomoles of L-asparagine formed per hour at 37°C.

this alternate substrate was elevated from 8.3 to 12.5 mM (fig 5). These findings suggest that 90% of the molecules of L-asparagine synthetase bind the inhibitor at their "L-glutamine-site" and have no residual affinity for L-glutamine, whereas the other 10% of the molecules of enzyme have escaped interaction and retain normal affinity for the amide. It can also be inferred from these results that the "ammonia site" is substantially intact after inhibition, but subtly altered in such a way that higher concentrations of ammonia are needed to saturate it. Alternatively, it is possible that the ammonia site can in fact utilize L-glutamine with the same avidity as the native enzyme but at ~ 10% of the V_{max} seen with ammonia, and also that no L-glutamine sites survive exposure to the comparatively large molar excess of inhibitor used. Evidence against this supposition is provided by the finding that 6-diazo-5-oxo-L-norleucine (DON; NSC–7365) can inhibit L-glutamine utilization by the isoxazole-treated enzyme to the same extent that it inhibits the native enzyme (85% at 1 mM).

In this regard, it is of interest that the utilization of ammonia by the "isoxazole enzyme" was found to be comparatively refractory to inhibition by the sulfhydryl reagent mucochloric acid (12), whereas inhibition of the utilization of L-glutamine was enhanced

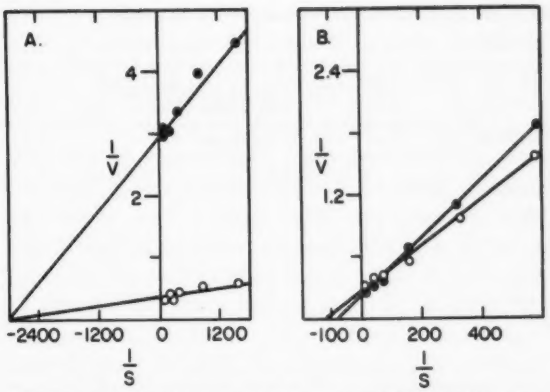

FIGURE 5.—Comparison of the apparent Michaelis constants for L-glutamine and ammonia of L-asparagine synthetase exposed to NSC-163501 and subjected to dialysis. Aliquots of a partially purified L-asparagine synthetase from L5178Y/AR leukemia (specific activity, 0.0058 IU/mg of protein) were exposed to NSC-163501 at a 1.0 mM concentration (●) or tris-HCl buffer (pH 7.4) (○) for 30 min at 25°C, and were then dialyzed for 6 hrs at 4°C against 4 liters of 0.1 M tris-HCl (pH 7.4) containing dithiothreitol (1.0 mM) and EDTA (0.5 mM). The Michaelis constants of these dialyzed enzyme preparations for L-glutamine and ammonia were then determined as described in the Materials and Methods section. 1/v represents the reciprocal of nanomoles of L-asparagine formed per hour at 37°C.

under the same condition. Conversely, the native enzyme is more susceptible to inhibition by mucochloric acid when ammonia, as opposed to L-glutamine, is the substrate. An analogously prepared "mucochloric acid enzyme" (Materials and Methods) was still fully susceptible to selective inhibition of amide transfer when exposed to the isoxazole at a concentration of 1 mM (table 3).

In order to determine whether the ability to inhibit L-asparagine synthetase was a general property of compounds with an isoxazole ring, several analogs of NSC–163501 were tested against the partially purified enzyme from murine tumor cells. The results of this study are presented in table 4 and warrant the conclusion that the isoxazole ring system must be associated with an α-aminocarboxylic acid for significant inhibitory activity to be expressed. Thus, although the structure-activity series is small, only NSC–163501 and its ring-hydroxylated congener NSC–176324 produced significant inhibition of tumoral L-asparagine synthetase at a concentration of 1 mM. When ibotenic acid,[14] an isoxazole of fungal origin, was incubated with the enzyme at a final concentration of 10 mM, the compound did inhibit L-asparagine synthetase to a significant degree. However, unlike NSC-163501, ibotenic acid interfered with the utilization of both L-glutamine and ammonia, so that the mechanism of this inhibit-

[14] Generously supplied by Dr. T. Takemoto, Pharmaceutical Institute, University of Tohoku, Sendai, Japan.

TABLE 3.—Effect of pretreatment of L-asparagine synthetase from L5178Y/AR leukemia with mucochloric acid or NSC-163501 on subsequent inhibition by the agents*

			Subsequent inhibition (%) by—			
	Residual activity (%): substrates		Mucochloric acid: substrates		NSC-163501: substrates	
Pretreatment	L-Glutamine	NH_4Cl	L-Glutamine	NH_4Cl	L-Glutamine	NH_4Cl
Saline ("native enzyme")	100	100	32	55	74	0
Mucochloric acid ("mucochloric enzyme")	43	32	24	43	72	0
Isoxazole ("isoxazole enzyme")	6	100	64	14	74	0

*"Isoxazole enzyme" and "mucochloric enzyme" were prepared by incubating a partially purified preparation of L-asparagine synthetase from L5178Y/AR leukemia (specific activity 0.03 IU/mg of protein) with NSC-163501 (1 mM) or mucochloric acid (2 mM) followed by dialysis as described in the Materials and Methods section. Dialyzed enzyme was then incubated with NSC-163501 or mucochloric acid in the presence of the other substrates of L-asparagine synthetase and the production of L-[4-^{14}C]asparagine was measured as described in the Materials and Methods section.

TABLE 4.—In vitro inhibition of L-asparagine synthetase from L5178Y/AR tumor by analogs of NSC-163501

Inhibitors*	Concentration (mM)	Inhibition (%) of L-asparagine synthesis	
		Substrates	
		L-Glutamine	Ammonia
NSC-163501	1.0	85	0
NSC-176324	1.0	79	0
3-Isoxazolecarboxamide, *N*-[3-(chloromercuri)-2-methoxypropyl]-5-methyl-	1.0	20	0
4-Isoxazolecarboxamide, *N*-[3-(chloromercuri)-2-methoxypropyl]-3,5-dimethyl-	1.0	0	0
5-Isoxazolecarboxamide, *N*-ethyl-3-methyl-	1.0	0	0
4-Isoxazolecarboxaldehyde, 3,5-dimethyl-	1.0	0	0
5-Isoxazolecarboxamide, *N*-(2-hydroxyethyl)-3-methyl-	1.0	0	0
5-Isoxazoleacetic acid, α-amino-3-hydroxy- (ibotenic acid)	1.0 10.0	26 78	21 72
5-Isoxazolidineacetic acid, α-amino-3-oxo, *erythro*- (tricholomic acid)†	1.0	0	0
3-Isoxazolecarboxamide-, 5-methyl-	1.0	0	0
3-Isoxazolecarboxamide, 5-methylthio-	1.0	0	0
Isoxazole, 5-methyl-	1.0	0	0

*Inhibitors were dissolved in water and neutralized with 1 N NaOH or HCl. Measurements of L-asparagine synthetase were conducted according to the methodology presented in the Materials and Methods section.

†Generously supplied by Dr. C. H. Eugster, Institute for Organic Chemistry, University of Zurich, Switzerland.

or is very likely different from that of NSC–163501.

Pursuant to these observations, experiments were carried out to determine whether the inhibitor molecule was altered, as for example, by an act of abortive catalysis, during its reaction with L-asparagine synthetase. For this purpose, the amino acid analyzer was employed to chromatograph scaled-up reaction mixtures containing NSC–163501 at 1×10^{-3} M and a concentration of partially purified enzyme 200 times that normally used. No evidence for the generation of a new compound was found in these studies. However, the use of the radioactive species (were it available) would add greater credibility to such preliminary negative results. Attempts were also made to demonstrate binding of NSC–163501 to a partially purified preparation of L-asparagine synthetase from L5178Y/AR leukemia (*see* Materials and Methods). These attempts were unsuccessful probably as a consequence of the low molarity of the enzyme used. Failure to detach the putatively bound inhibitor with 1 M acid and alkali may indicate that stronger conditions will be needed or that methods more sensitive than the amino acid analyzer will have to be used. Parenthetically, experiments designed to demonstrate decomposition of NSC–163501 by exposure to extremes of pH followed by automated amino acid analysis, as well as assessment of inhibitory potency towards L-asparagine synthetase, showed no evidence of breakdown or loss of inhibitory activity.

Lastly, although only a limited supply of NSC–163501 was available, attempts were made

to determine whether the drug was able to inhibit L-asparagine synthetase in vivo. Towards this end, adult male BDF_1 mice bearing sc implants of L5178Y/AR leukemia were given ip doses of 5 or 25 mg/kg of the antibiotic and were killed 4 hours later; the specific activity of L-asparagine synthetase was measured in their pancreata and tumors. Animals treated with NSC–124412 at doses of 5 or 100 mg/kg were included for purposes of comparison. The results of this study, presented in table 5, point to the fact that the higher dose of NSC–163501 exerted a powerful inhibitory action on the enzyme from both sources only when L-glutamine was used as the amide donor. Even at the lower dose used, the drug produced significant inhibition of both L-asparagine synthetases. Since the instantaneous molarity achieved in vivo by the lower dose would approximate 2×10^{-5} M, and inasmuch as this concentration of drug exerted a lesser inhibition in vitro (table 1), it can be concluded that a certain degree of concentrative uptake had taken place. It is noteworthy that, in vivo, NSC–163501 was a more effective inhibitor on a molar basis than NSC–124412 in both of the organs examined; its potency was especially pronounced in the case of the L5178Y tumor.

The synthesis of L-asparagine is only one of a number of enzymatic reactions involving amide donation by L-glutamine. It is therefore relevant to inquire whether these analogous reactions are also subject to inhibition by NSC–163501. Experiments to answer this question will be summarized in a subsequent paper.

TABLE 5.—In vivo effect of NSC-163501 on L-asparagine synthetase from tumor and pancreas of mice bearing L5178Y/AR leukemia*

		Inhibition (%) of L-asparagine synthetase from—			
		Tumor		Pancreas	
		Substrates		Substrates	
Drug	Dose (mg/kg)	L-Glutamine	Ammonia	L-Glutamine	Ammonia
NSC-163501	25.0	77†	0	77†	0
	5.0	57†	0	37†	0
NSC-124412	100.0	34†	0	74†	65†
	5.0	0	0	0	0

*Groups of 10 BDF_1 mice bearing L5178Y/AR tumor were injected ip with saline or with 25 or 5 mg/kg of NSC-163501 or with 100 or 5 mg/kg of NSC-124412. Pancreas and tumor were collected 4 hours later, homogenized, and assayed for L-asparagine synthetase activity as described in the Materials and Methods section. Percentage inhibition has been calculated by comparison with identical analyses carried out on saline-treated groups. Measurements were made in triplicate.

†$P < 0.01$.

LIST OF COMPOUNDS

(αS,4S,5R)-α-Amino-3-chloro-4-hydroxy-4,5-dihydro-5-isoxazoleacetic acid: NSC-176324; U-43,795

L-(αS,5S)-α-Amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid: NSC-163501; U-42,126

L-Asparaginase: NSC-109229; CAS reg. No. 9015-68-3

5-Chloro-4-oxo-L-norvaline: NSC-124412; CAS reg. No. 15148-06-8; levulinic acid, 2-amino-5-chloro-, hydrochloride, L-; CONV

5-Isoxazoleacetic acid, α-amino-3-hydroxy-: ibotenic acid

4-Isoxazolecarboxaldehyde, 3,5-dimethyl-

4-Isoxazolecarboxamide, *N*-[3-(chloromercuri)-2-methoxypropyl]-3,5-dimethyl-: NSC-58765

3-Isoxazolecarboxamide, *N*-[3-(chloromercuri)-2-methoxypropyl]-5-methyl-: NSC-58764; CAS reg. No. 5580-28-9

5-Isoxazolecarboxamide, *N*-ethyl-3-methyl-: NSC-66023

5-Isoxazolecarboxamide, *N*-(2-hydroxyethyl)-3-methyl-: NSC-66018; CAS reg. No. 21706-42-3

3-Isoxazolecarboxamide, 5-methyl-: NSC-18369; CAS reg. No. 3445-52-1

3-Isoxazolecarboxamide, 5-methylthio-: NSC-18367

Isoxazole, 5-methyl-: NSC-52269; CAS reg. No. 5765-44-6

5-Isoxazolidineacetic acid, α-amino-3-oxo, *erythro*-: CAS reg. No. 2644-49-7; tricholomic acid

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Antitumor Activity of Some Acylhydrazines [1,2]

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SUMMARY

A series of analogs of 1-acetyl-2-picolinoylhydrazine were prepared and tested against the Sarcoma 180, L1210 leukemia, and Walker 256 carcinosarcoma systems. Significant activity was observed only in the latter system. Based on the analogs which showed activity, the following observations were made: (*a*) The picolinic acid residue in 1-acetyl-2-picolinoylhydrazine may be replaced by others such as pipecolic, pyrazinoic, benzoic, and several substituted benzoic acid residues with partial retention of activity. (*b*) The acetyl group at N_1 can be absent altogether or can be replaced by selective acyl groups without loss of activity.

[Cancer Chemother Rep 58:803–810, 1974]

In a program set up at our laboratories for the evaluation of synthetic compounds for antitumor activity, it was observed that 1-acetyl-2-picolinoylhydrazine (NSC-68626) (fig 1) had significant activity against Walker 256 (W256) carcinosarcoma. This compound had since been approved for and is currently in clinical trials (1–3).

To define more clearly the structure-activity relationships in this group and to uncover more active members, we initiated a program to synthesize analogs of this compound and to evaluate their antitumor activity. In spite of the relatively simple chemistry involved, it was felt that obtaining such correlative information would justify the efforts in this direction.

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[2] Supported by Public Health Service contract PH43-68-45 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

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The compounds in this study can be divided into three groups based on the site and nature of the structural alteration. These involve (*a*) the heterocyclic system, (*b*) the hydrazine system, and (*c*) the acyl function. Members of each group are listed with their physical and analytic data in table 1.

The Heterocyclic System

This group includes acetylhydrazides of carboxylic acids of pyridine, pyrazine, benzene, etc (compound Nos. 1–17).

The Hydrazine System

Various acylhydrazines (ie, without the N_1 substituent) and methyl-substituted acylhydrazines are included in this group (Nos. 18–36). Picolinoylhydrazones of three carbonyl compounds (Nos. 37–39) were also tested to see if they might have longer activity (4). In view of the known antitumor activity of bisguanylhydrazones of methylglyoxal and analogs (5), two such compounds are included here (Nos. 40 and 41).

The Acyl Function

Variants in which the acetyl group of the parent compound is replaced by other acyl groups are represented by Nos. 42–51.

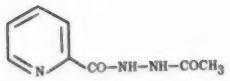


FIGURE 1.—Structure of 1-acetyl-2-picolinoylhydrazine.

RESULTS

All compounds were tested according to the protocols of Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, in the standard three-tumor screen (6) which is comprised of Sarcoma 180 (S180) and L1210 leukemia in mice and W256 carcinosarcoma in rats. Since neither the parent compound nor its analogs were active in the S180 or L1210 systems, only the results of the W256 system are considered here. Instead of presenting them in their raw form, the data have been arranged to provide a better means for comparison. Thus, the maximum tolerated dose (LD10), the minimum effective dose (T/C-40), the therapeutic index, and the extent of inhibition at the LD10 are given in a tabular form. Table 1 shows all the analogs tested in this study and table 2 shows all those showing significant activity in the W256 system.

DISCUSSION

To arrive at a meaningful structure-activity correlation, several possible hypotheses were examined. Although at this point none can give a satisfactory explanation, it would be useful to provide a brief discussion of each.

During the early phase of the work it was observed that the parent compound gave a deep blue color when treated with and exhibited a strong affinity for Fe^{3+} ion. The isomeric compounds Nos. 2 and 3, which were inactive, did not share these properties, while related compounds Nos. 5 and 6, which showed similar affinity, were active. In addition, the activity (as well as the affinity for Fe^{3+} ion) of 1-acetyl-2-picolinoylhydrazine was lost when it was quaternized (No. 7) or converted to the N-oxide (No. 8). These observations suggested possible correlation between metal-binding ability and activity along lines suggested for 2-formylpyridinethiosemicarbazone (7). However, this proved to be untenable when a number of compounds which did not possess this metal-binding ability were found to be active. For instance, the benzoyl (No. 9) and pipecoloyl (No. 16) derivatives showed excellent activity comparable to that of the parent compound.

Since acetohydrazide was shown to be a major metabolite of 1-acetyl-2-isonicotinoylhydrazine (8,9) (No. 3), it was felt that this compound might also arise from 1-acetyl-2-picolinoylhydrazine (No. 1) and hence be the actual active agent. In support of this, acetohydrazide (No. 25) was found to be highly active. However, a lack of activity in compound No. 3 itself and in several closely related compounds shows that this view is also untenable.

Finally, a comparison of the effective doses of the N-acetyl acylhydrazines and those of unsubstituted acylhydrazines (such as compounds Nos. 1 and 18, 5 and 22, 9 and 29, 16 and 24, and 17 [which was inactive] and 25) suggests that hydrolysis of the N_1 acetyl function might be a prerequisite for activity.

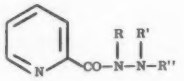
Thus, although no clear-cut generalizations can be made at this time regarding the minimum structural requirements for activity, a number of variables have been examined and several active compounds uncovered. Such hitherto unsuspected compounds as acetohydrazide and N_1-acetyl-N_2-benzoylhydrazine have been shown to have significant activity in this tumor system. The activity of the parent compound in man seems to warrant further studies in this area (2,3).

TABLE 1.—Analogs of 1-acetyl-2-picolinoylhydrazine tested in the present study

Compound No.	NSC No.	Structure	Melting point*	Formula	Analysis						Reference No.	CAS reg. No.
					Calculated				Found			
					C	H	N	O	H	N		
		Class A: $R–NH–NH–COCH_3$ **R=**										
1	68626	Picolinoyl	—	$C_8H_9N_3O_2$	—	—	—	—	—	—	10	17433-31-7
2	73289	Nicotinoyl	142–143	$C_8H_9N_3O_2$	53.62	5.06	23.45	53.83	5.35	23.28		7254-31-1
3	36084	Isonicotinoyl		$C_8H_9N_3O_2$	—	—	—	—	—	—	11	—
4	101635	2–Pyridyl–C (=NH)–	231–232	$C_8H_{10}N_4O$	53.92	5.66	31.45	53.86	5.84	31.68		25433-37-8
5	82129	Pyrazinoyl	215–216	$C_7H_7N_4O_2$	46.66	4.48	31.70	46.56	4.58	31.45		—
6	84496	Quinaldoyl	—	$C_{12}H_{11}N_3O_2$	—	—	—	—	—	—	12	—
7	123504	Methiodide of NSC-68626	170–171	$C_9H_{12}IN_3O_2$	33.68	3.77	13.08	33.65	3.88	13.05		—
8	126775	N-oxide of NSC-68626	215–216	$C_9H_9N_3O_3$	49.23	4.65	21.53	48.95	4.67	21.59		—
9	84587	Benzoyl	—	$C_9H_{10}N_2O_2$	—	—	—	—	—	—	13	14331-27-2
10	93071	2-Aminobenzoyl	—	$C_9H_{11}N_3O_2$	—	—	—	—	—	—	14	28864-26-8
11	91846	2-Hydroxybenzoyl	187–188	$C_9H_{10}N_2O_3$	55.66	5.19	14.43	11.64	5.21	14.34		20349-50-2
12		4-Hydroxybenzoyl	—	$C_9H_{10}N_2O_3$	—	—	—	—	—	—	15	—
13		4-Chlorobenzoyl	—	$C_9H_9ClN_2O_2$	—	—	—	—	—	—	16	—
14		4-Nitrobenzoyl	—	$C_9H_9N_3O_4$	—	—	—	—	—	—	16	—
15		3-Nitrobenzoyl	—	$C_9H_9N_3O_4$	—	—	—	—	—	—	17	—
16	114811	Pipecoloyl (HCl salt)	267–268	$C_8H_{15}N_3O_2$ ·HCl	43.35	7.27	18.94	43.65	7.34	18.74		—
17	42939	Acetyl	—	$C_4H_8N_2O_2$	—	—	—	—	—	—	13	3148-73-0
		Class B-1: $R–NH–NH_2$ **R=**										
18	81875	Picolinoyl	—	$C_8H_9N_3O$	—	—	—	—	—	—	18	—

TABLE 1.—Analogs of 1-acetyl-2-picolinoylhydrazine tested in the present study—Continued

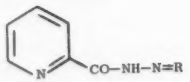
Compound No.	NSC No.	Structure	Melting point*	Formula	Analysis						Reference No.	CAS reg. No.
					Calculated				Found			
					C	H	N	O	H	N		
19	36088	Nicotinoyl	—	$C_6H_7N_3O$	—	—	—	—	—	—	19	553-53-7
20	9659	Isonicotinoyl	—	$C_6H_7N_3O$	—	—	—	—	—	—	19	54-85-3
21	101642	2–Pyridyl–C (=NH)–	—	$C_6H_5N_4$	—	—	—	—	—	—	20	1005-02-3
22	88100	Pyrazinoyl	—	$C_5H_6N_4O$	—	—	—	—	—	—	21	768-05-8
23	126776	N-oxide of NSC-81875	—	$C_6H_7N_3O_2$	—	—	—	—	—	—	22	—
24	123501	Pipecoloyl (HCl salt)	207–209	$C_6H_{12}N_3O$ ·HCl	40.10	7.85	23.37	39.84	8.15	23.10		—
25	53155	Acetyl	—	$C_2H_6N_2O$	—	—	—	—	—	—	13	1068-57-1
26	59489	Quinaldoyl	—	$C_{10}H_9N_3O$	—	—	—	—	—	—	23	5382-44-5
27	123503	2–Quinoxalinyl–CO–	—	$C_9H_5N_4O$	—	—	—	—	—	—	24	—
28	28895	2–Indolyl–CO–	—	$C_9H_9N_3O$	—	—	—	—	—	—	18	5055-39-0
29	644	Benzoyl	—	$C_7H_8N_2O$	—	—	—	—	—	—	13	613-94-5



	R=	R'=	R''=									
30	126767	CH_3	H	H	107–108	$C_7H_9N_3O$	55.61	6.00	27.80	55.51	5.91	27.98		—
31	129619	H	CH_3	H	61–62	$C_7H_9N_3O$	55.61	6.00	27.80	55.52	6.07	27.80		—
32	126768	CH_3	CH_3	H	47–49	$C_8H_{11}N_3O$	58.16	6.71	25.44	58.11	6.73	25.45		—
33	129377	H	CH_3	$COCH_3$	167–168	$C_9H_{11}N_3O_2$	55.95	5.74	21.74	55.81	5.80	21.95		—

34	126770	CH_3	CH_3	$COCH_3$	Oil	$C_{10}H_{12}N_2O_2$	57.96	6.32	20.23	57.73	6.41	20.57		—
35	112150	H	$COCH_3$	$COCH_3$	100–101	$C_{10}H_{11}N_3O_3$	54.29	5.01	19.00	54.43	5.21	19.11		—
36	111544	$COCH_3$	$COCH_3$	$COCH_3$	84–86	$C_{12}H_{13}N_3O_4$	54.75	4.98	15.96	54.71	5.23	16.06		—

Class B-2:



R=

37	90914	4-$(CH_3)_2N$-C_6H_4-CH=	211–212	$C_{15}H_{16}N_4O$	67.14	6.01	20.88	67.03	6.09	20.65		—
38	106460	2-Pryidyl-CH=	165–167	$C_{11}H_{10}N_4O$	—	—	—	—	—	—	25	15017-24-0
39	106457	CH_3-CO-$C(CH_3)$=	189–191	$C_{10}H_{11}N_3O_2$	58.53	5.40	20.48	58.35	5.46	20.70		—
40	98913		250–251	$C_{15}H_{14}N_6O_2$	53.58	3.60	8.32	53.61	3.42	8.50		—

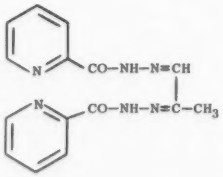
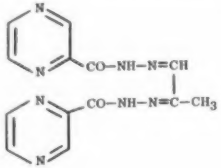
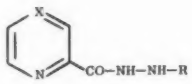


TABLE 1.—Analogs of 1-acetyl-2-picolinoylhydrazine tested in the present study—Continued

Compound No.	NSC No.	Structure	Melting point*	Formula	Analysis: Calculated C	H	N	O	Found H	N	Reference No.	CAS reg. No.
41	106459		>300	$C_{13}H_{12}N_6O_2$	50.00	3.88	35.60	50.21	4.00	35.72		—

Class C:



	R=	X=									
42	103351	Formyl	CH	152–153	$C_7H_7N_3O_2$	50.91	4.27	25.45	50.83	4.23	25.60	—
43	90922	Propionyl	CH	140–141	$C_9H_{11}N_3O_2$	55.95	5.74	21.75	56.19	5.83	21.77	—
44	90923	N–Butyryl	CH	124–125	$C_{10}H_{13}N_3O_2$	57.96	6.32	20.28	57.87	6.36	20.22	—
45	90919	Bromoacetyl	CH	141–142	$C_8H_8BrN_3O_2$	37.24	3.12	16.28	37.35	3.21	16.42	—
46	90912	Dichloroacetyl	CH	138–139	$C_8H_7Cl_2N_3O_2$	38.70	2.84	16.93	38.83	2.91	16.82	—
47	95396	Methanesulfonyl	CH	150–151	$C_7H_9N_3O_4S$	39.07	4.22	19.53	39.02	4.19	19.34	—

48	90913	Benzoyl	CH	—	$C_{12}H_{11}N_3O_2$	—	—	—	—	—	—	26	—
49	90924	Picolinoyl	CH	—	$C_{12}H_{10}N_4O_2$	—	—	—	—	—	—	27	840-79-9
50	103345	Formyl	N	—	$C_6H_6N_4O_2$	—	—	—	—	—	—	28	—
51	95395	Pyrazinoyl	N	—	$C_{10}H_8N_6O_2$	—	—	—	—	—	—	24	—

*Melting points were taken on Fisher-Johns apparatus and are uncorrected.

TABLE 2.—W256 activity of analogs of 1-acetyl-2-picolinoylhydrazine

Compound No.*	NSC No.	Route†	LD10 (mg/kg)	T/C-40‡ (mg/kg)	TI§	TWI¶ (%)
1	68626	ip	250	76	3.3	93
		im, twice daily	350	80	4.4	100
		iv, twice daily	400	56	7.1	97
		sc, twice daily	350	80	4.4	97
		oral	290	60	4.8	90
5	82129	ip	500	125	4.0	97
		oral	300	80	3.8	98
6	84496	ip	200	130	1.5	82
9	84587	ip	300	75	4.0	94
		oral	290	78	3.7	94
13		ip	310	190	1.6	75
14		ip	300	190	1.7	70
15		ip	250	150	1.7	75
16	114811	ip	580	240	2.4	90
		oral	680	270	2.6	93
18	81875	ip	110	54	2.6	92
		oral	120	60	2.0	85
22	88100	ip	180	85	2.1	91
		oral	200	90	2.2	95
24	123501	ip	440	170	2.6	96
		oral	660	240	2.5	95
25	53155	ip	160	52	3.0	94
28	28895	ip	350	330	1.1	64
29	644	ip	110	80	1.4	80
35	112150	ip	400	125	3.2	94
39	106457	ip	230	100	2.3	85
42	103351	ip	285	200	1.4	72
43	90922	ip	250	120	2.0	82
44	90923	ip	135	85	1.5	88
46	90912	ip	170	100	1.7	94
48	90913	ip	195	100	2.0	94
49	90924	ip	175	100	1.8	89
50	103345	ip	540	400	1.3	71
51	95395	ip	340	240	1.4	82

*Numbers refer to those used in table 1.

†Compounds were administered as solutions in physiologic saline or, if insoluble, as fine suspensions in 0.5% Tween-80.

‡Dose at which the tumor weight of treated vs control animals was 40% or less, ie, minimum effective dose.

§Ratio of maximum tolerated dose (LD10) vs minimum effective dose (T/C-40).

¶Percent inhibition of tumor greater at maximum tolerated dose.

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Altered Sensitivity of Hematopoietic Stem Cells to 5-Fluorouracil (NSC-19893) Following Endotoxin (NSC-189681), Cyclophosphamide (NSC-26271), or Irradiation [1,2,3]

A. C. Eaves and W. R. Bruce [4,5,6]

SUMMARY

Hematopoietic cells that form colonies in the spleen (CFU-S) or in culture (CFU-C) from the marrow of endotoxin-treated mice become markedly sensitive to a 6-hour in vivo exposure to 2.5 mg of 5-fluorouracil (5-FU). It was therefore suggested that short exposures to 5-FU (2.5 mg given 6 hours before the animals were killed) could be used to follow changes in the sensitivity of CFU-S and CFU-C after several treatments which might stimulate the proliferation of marrow colony-forming cells. After 25 μg of endotoxin was given, a maximum reduction (~100-fold) in the number of colony-forming cells in 5-FU-treated mice occurred at 36 hours, with recovery to starting values by 72 hours. Similar changes in sensitivity to 5-FU were also seen in mice whose marrows were undergoing hematopoietic regeneration after 3 mg of cyclophosphamide or 150 rads of total-body irradiation. These results indicate that agents which perturb the marrow may be quantitatively examined on the basis of their ability to alter the sensitivity of hematopoietic progenitor cells to chemotherapeutic agents.

[Cancer Chemother Rep 58:813–820, 1974]

Hematopoietic progenitor cells that form colonies in the spleen (colony-forming units, spleen [CFU-S]) or in culture (colony-forming units, culture [CFU-C]) are more sensitive to the in vivo administration of many chemotherapeutic agents when they are in a state of active proliferation (1–3). Studies supporting this view initially demonstrated the altered sensitivity of CFU-S regenerating in lethally irradiated recipients of a graft of normal marrow cells. To exclude the possibility that the transplantation procedure itself might have introduced artifacts leading to an increased sensitivity unrelated to cycle state, a different proliferative stimulus was sought (3). Stem cells from mice injected with endotoxin 24 hours previously were found to be sensitive to in vitro exposure to high specific-activity tritiated thymidine and therefore to be actively proliferating. The stem cells of such endotoxin-treated mice were also found to be markedly sensitive to in vivo administration of a number of chemotherapeutic agents, thus supporting the concept that chemotherapeutic sensitivity was dependent on the proliferative state but independent of the stimulus used to

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[2] Supported by a grant from the National Cancer Institute of Canada.

[3] 5-Fluorouracil: CAS reg. No. 51-21-8; 5-FU. Supplied by Hoffmann-LaRoche, Ltd., Montreal, Canada.

Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

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[5] We wish to thank V. Eng and K. Ryckman for their expert technical assistance.

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initiate proliferation. Possible mechanisms by which endotoxin induces proliferation of hematopoietic stem cells are currently being investigated (4).[7]

In this report, we have examined two other agents which were expected to induce proliferation of hematopoietic progenitor cells in vivo—cyclophosphamide and sublethal whole-body irradiation—and we have compared them to endotoxin. The hematopoietic changes induced by these three different agents were measured in terms of the in vivo sensitivity they conferred on both CFU-S and CFU-C to 5-fluorouracil (5-FU). A 6-hour in vivo exposure to 2.5 mg of 5-FU was chosen to define the kinetics of this response rather than the 30-minute in vitro exposure to high specific-activity tritiated thymidine used previously (3). Such use of 5-FU permitted measurement of hundredfold changes in sensitivity compared to the twofold changes measured using in vitro tritiated thymidine. This approach provides a quantitative in vivo method for (*a*) measuring the effect of agents that alter the proliferative status of hematopoietic progenitor cells, and (*b*) measuring the marrow toxicity associated with sequential chemotherapy or chemotherapy following immunotherapy.

MATERIALS AND METHODS

Mice

Female AKR mice, 6–8 weeks of age, were obtained from Jackson Laboratory, Bar Harbor, Maine, and used when 10–16 weeks old. Whole-body irradiation of mice, for both the spleen colony assay and the kinetic studies after administration of 150 rads, was carried out in a ^{137}Caesium unit designed by Cunningham, Bruce, and Webb (5).

Preparation of Marrow Suspensions

Mice were killed by cervical dislocation and the skin was removed from the abdomen and legs. The femurs from three or four mice were aseptically dissected and adjacent muscle tissue was removed. Each femur was then flushed out using a 23-gauge needle and approximately 3 ml of alpha tissue culture medium (Flow Laboratories, Rockville, Md) supplemented with 10% horse serum (Woodlyn Farms, Guelph, Ontario). Single-cell suspensions were prepared by syringing suspensions up and down six times through an 18-gauge needle, allowing them to settle for 3 minutes, and discarding the sediment. The cell suspensions were prepared at a concentration of one femur/ml (approximately 2×10^7 nucleated cells/ml) and appropriately diluted for either injection into lethally irradiated mice (CFU-S assay) or plating in culture medium (CFU-C assay).

Assays for Colony Formation

CFU-S were measured by the method of Till and McCulloch (6). Marrow suspensions were injected via the tail vein into mice that had received 950 rads of whole-body irradiation. Nine days later the mice were killed, the spleens were removed and preserved in Bouin's fixative, and macroscopic surface colonies were counted.

CFU-C were measured by the methyl cellulose culture method of Worton et al (7) based on the method of Pluznik and Sachs (8) and Bradley and Metcalf (9). Each replicate 35-mm diameter petri plate (Falcon Plastics, Oxnard, Calif) contained 0.4 ml of 2% methyl cellulose (Dow Chemical Co., 4000 CPS) in alpha medium, 0.2 ml of horse serum, 0.2 ml of L-cell conditioned medium as a source of colony-stimulating activity (10), and 0.2 ml of an appropriate dilution of bone marrow cells prepared as described above. Incubation was at 37° C in a humidified atmosphere of 10% CO_2 in air for 7 days. Colonies with more than 20 cells were scored with an inverted microscope at a magnification of ×35.

Administration of Reagents

5-FU and cyclophosphamide were diluted in 0.9% saline. The endotoxin used was a preparation of lipopolysaccharide B from *Sal-*

[7] Eaves AC, and Bruce WR. In vitro production of colony-stimulating activity. II. Cellular interaction between adherent and non-adherent mouse peritoneal cells exposed to concanavalin A. Manuscript in preparation.

Eaves AC, and Bruce WR. In vitro production of colony-stimulating activity. III. Exposure of human peripheral blood leukocytes to endotoxin, phytohemagglutinin and concanavalin A. Manuscript in preparation.

monella typhosa. It was suspended in 0.9% saline and stored at −20° C until just prior to use. All reagents were injected via the tail vein in a volume of 0.5 ml.

RESULTS

Survival as a Function of the Interval Between 5-FU Administration and Time of Assay

In earlier studies (3), a dose of 2.5 mg of 5-FU was found to result in a hundredfold reduction in CFU-S and CFU-C in endotoxin-treated mice compared to untreated mice. However, these studies had been done on mice treated with 5-FU 24 hours before the animals were killed for CFU-S and CFU-C measurements. To clearly assess the changes in sensitivity to 5-FU in endotoxin-treated mice, a shorter exposure time was required. Therefore, to determine the minimum time necessary to obtain maximum killing by 5-FU, an experiment was performed in which normal and endotoxin-treated mice were killed for CFU-S and CFU-C measurements at various intervals after the injection of 2.5 mg of 5-FU.

The results (fig 1) show that the number of CFU-S detected in mice that did not receive endotoxin gradually decreased in an exponential fashion over the 24-hour observation period. CFU-S in mice that had received endotoxin 24 hours before 5-FU were much more sensitive to 5-FU during the first 4–6 hours following 5-FU; thereafter, they declined at the same rate as observed in the controls. CFU-C in control mice were more sensitive to 5-FU initially and then declined more slowly in a fashion similar to CFU-S. CFU-C in endotoxin-treated mice were reduced to undetectable levels 1 hour after a dose of 2.5 mg of 5-FU. On the basis of these results a 6-hour exposure period to 2.5 mg of 5-FU was used for subsequent studies.

Endotoxin Followed by 6-Hour Exposure to 5-FU

The kinetics of the sensitivity of CFU-S and CFU-C to a 6-hour exposure of 2.5 mg of 5-FU was then determined as a function of time after endotoxin administration. A large number of mice were each injected with 25 μg of endotoxin at time 0 and divided into an experimental and a control group. At intervals thereafter, three mice from the experimental group were injected with 2.5 mg of 5-FU and 6 hours later were killed; three mice from the control group were also killed at the same time but did not receive 5-FU. At the time of death, the femoral contents of each group were made into a single cell suspension, a nucleated cell count was done, and the cells were either injected into lethally irradiated mice for CFU-S determination or plated for CFU-C assay.

The results (fig 2) show that endotoxin caused a twofold decrease in marrow cellularity (upper panel, closed symbols) that was not affected by a 6-hour exposure to 5-FU (upper panel, open symbols). The effect of 5-FU alone on the nucleated cell count and CFU populations in mice that did not receive endotoxin can be seen by comparing the time 0 values for mice not given 5-FU (closed symbols, vertical axis) with the values for mice given a 6-hour exposure to 2.5 mg of 5-FU (open symbols, vertical axis). 5-FU treatment alone had little effect on the nucleated cell count, although it caused a twofold decrease in CFU-S and a fivefold decrease in CFU-C. As a function of time after endotoxin, the total number of CFU-S per femur in endotoxin-treated mice remained essentially unchanged (closed symbols); however, the number of CFU-S able to survive a 6-hour exposure to 5-FU progressively decreased to reach a minimum (approximately 1% of starting value) at 36 hours (open symbols). Similar results were seen when the marrow samples were assayed for CFU-C (lower panel). Seventy-two hours after endotoxin was given, the sensitivity of stem cells to 5-FU had returned to levels seen in mice that had not received endotoxin (time 0 values). Thus, using a 6-hour exposure to 5-FU, the pattern of alteration in sensitivity of CFU-S and CFU-C to 5-FU induced by endotoxin treatment could be delineated.

Cyclophosphamide Followed by 6-Hour Exposure to 5–FU

An experiment of the same design was also carried out using cyclophosphamide instead of endotoxin. This was done to determine if a similar pattern of CFU sensitivity occurred

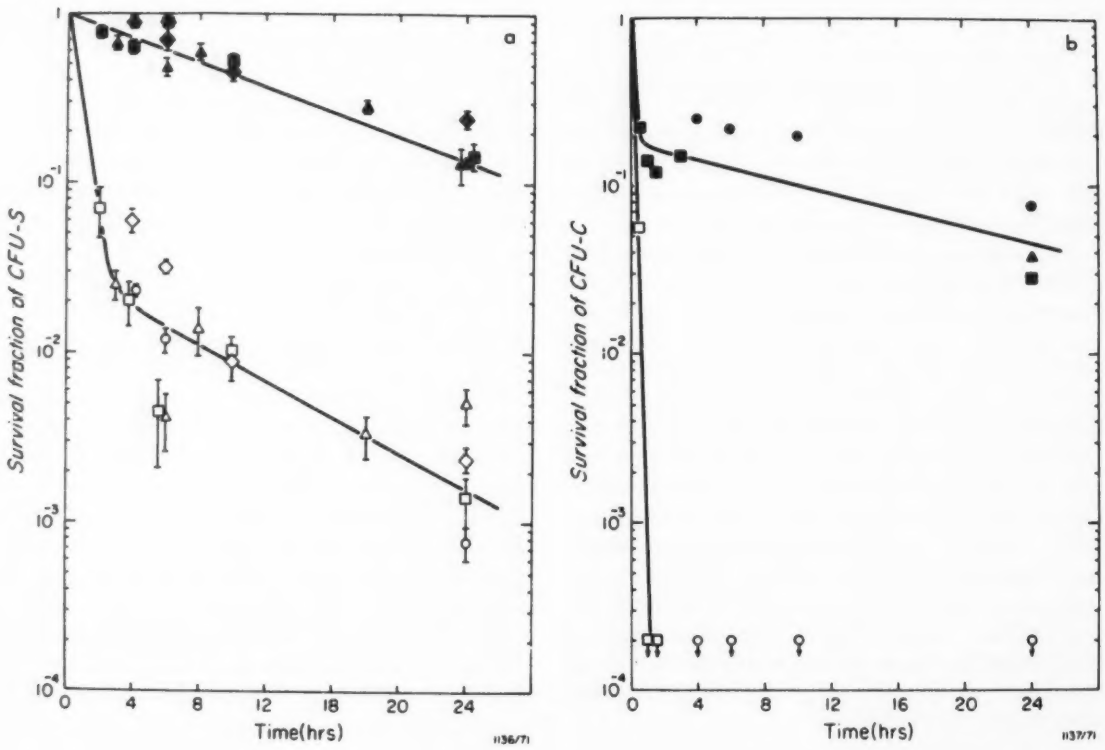

FIGURE 1.—The reduction in CFU-S and CFU-C as a function of time after 5-FU administration. Mice were injected with 25 μg of endotoxin and 24 hours later were given 2.5 mg of 5-FU. At various times after the 5-FU, groups of mice were killed and their femurs assayed for (*a*) CFU-S and (*b*) CFU-C. Mice that received endotoxin and 5-FU are represented by open symbols; control mice that received 5-FU only are represented by closed symbols. Different symbols are used for separate experiments. Each symbol depicting a CFU-S value represents the mean spleen colony counts from 15 or more spleens and the confidence intervals shown are 1 standard error of the mean; each symbol depicting a CFU-C value represents the mean number of colonies on two replicate plates.

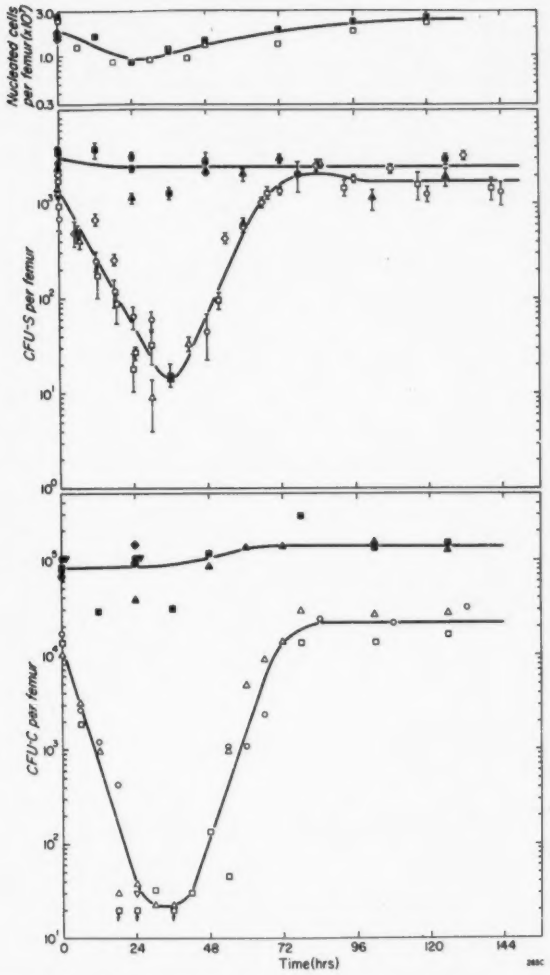

FIGURE 2.—Marrow sensitivity to 5-FU as a function of time after endotoxin. Mice were injected with 25 μg of endotoxin; at various intervals thereafter they were killed and the number of nucleated cells, CFU-S, and CFU-C were determined per femur (closed symbols). Other mice similarly treated with endotoxin were injected with 2.5 mg of 5-FU 6 hours before death and the same parameters were measured (open symbols). The origin of the value for each symbol is as described in fig 1.

following perturbation of the marrow with a chemical agent different from endotoxin. In this case mice were given a single intravenous injection of 3 mg of cyclophosphamide and groups of six mice were killed at intervals thereafter; 6 hours before death three mice in each group of six received 2.5 mg of 5-FU.

The results (fig 3) demonstrate that 3 mg of cyclophosphamide alone reduced the number of CFU-S from a control value of 3000 CFU-S per femur to approximately 400 CFU-S per femur by 24–36 hours (middle panel). Some of this decrease could have been due to the general decrease in marrow cellularity which also occurred regardless of 5-FU treatment (upper panel). In the remaining CFU-S that

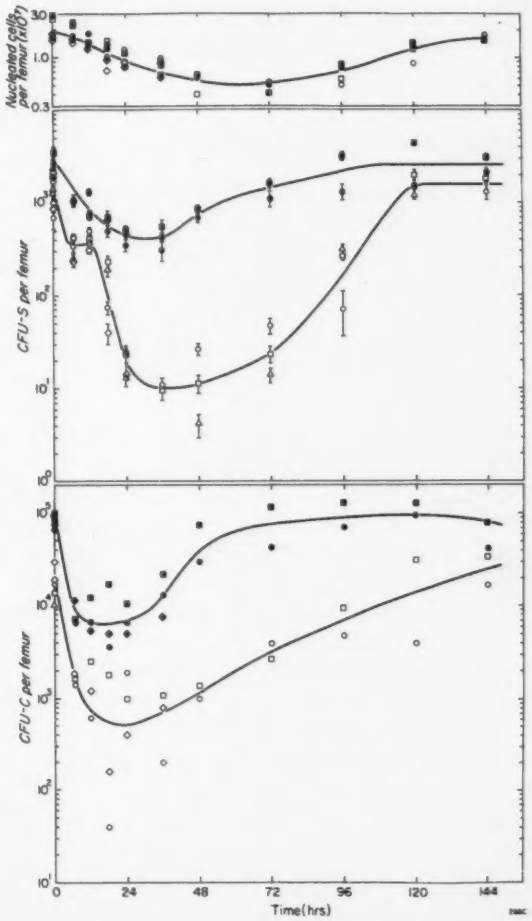

FIGURE 3.—Marrow sensitivity to 5-FU as a function of time after cyclophosphamide. Mice were injected with 3 mg of cyclophosphamide; at various intervals thereafter they were killed and the number of nucleated cells, CFU-S, and CFU-C were determined per femur (closed symbols). Other mice similarly treated with cyclophosphamide were injected with 2.5 mg of 5-FU 6 hours before death and the same parameters were measured (open symbols). The origin of the value for each symbol is as described in fig 1.

were not killed by cyclophosphamide, increased sensitivity to 5-FU was apparent within the first 24 hours. Compared to normal marrow of mice that did not receive cyclophosphamide, 5-FU reduced the numbers of CFU-S by a factor of only 2 (time 0 values); by 36 hours the same 5-FU treatment reduced the CFU-S population from 400 to ten per femur, a forty-fold reduction. The sensitivity of CFU-S to 5-FU then slowly returned to starting values by 120 hours after cyclophosphamide administration. Although not as marked as for CFU-S, the sensitivity of CFU-C to 5-FU was also increased after cyclophosphamide and showed similar kinetics (lower panel).

Gamma Irradiation Followed by 6-Hour Exposure to 5-FU

To follow the changes in 5-FU sensitivity after perturbation of the marrow by a physical cytotoxic agent, mice were given whole-body irradiation of 150 rads. The femoral content of nucleated cells, CFU-S, and CFU-C were then measured either with or without a 6-hour exposure to 2.5 mg of 5-FU as a function of time after the irradiation.

It can be seen (fig 4) that the number of nucleated cells per femur again decreased in all mice (upper panel). The total number of CFU-S (middle panel) was also reduced by the irradiation, from a control value of 3000 to approximately 300 CFU-S per femur present 36 hours later. However, compared to the twofold effect in reducing CFU-S numbers in nonirradiated mice, CFU-S present at 36 hours after irradiation were subject to a 45-fold reduction in number by 5-FU. This enhanced sensitivity of CFU-S to 5-FU returned to starting values by 96–120 hours after irradiation. Changes in the sensitivity of CFU-C to 5-FU, although not as great as seen in CFU-S, showed similar kinetics following irradiation (lower panel).

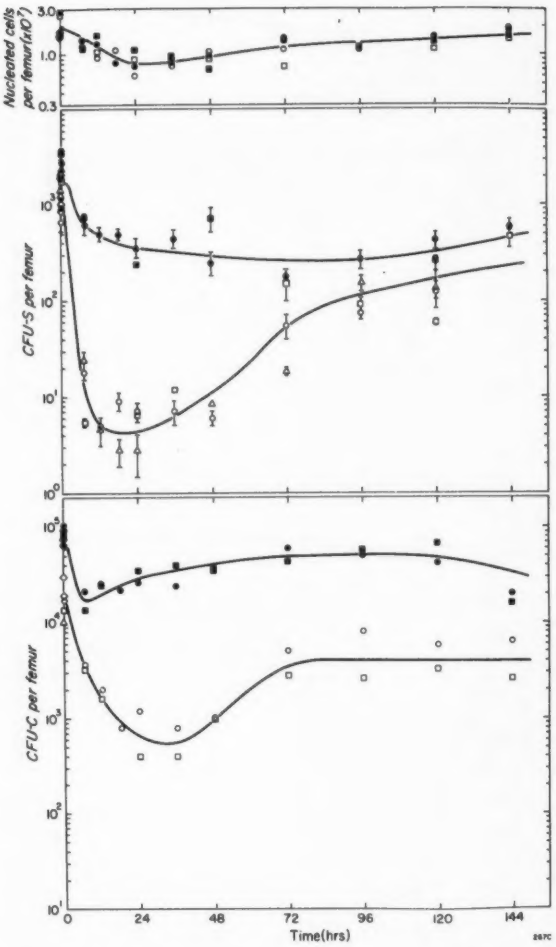

FIGURE 4.—Marrow sensitivity to 5-FU as a function of time after irradiation. Mice were exposed to 150 rads; at various intervals thereafter they were killed and the number of nucleated cells, CFU-S, and CFU-C were determined per femur (closed symbols). Other mice similarly irradiated were injected with 2.5 mg of 5-FU 6 hours before death and the same parameters were measured (open symbols). The origin of the value for each symbol is as described in fig 1.

DISCUSSION

These studies have examined the sensitivity of hematopoietic colony-forming cells to 5-FU as a function of time after a single treatment with either endotoxin, cyclophosphamide, or gamma radiation. It was found that mice given these three different treatments revealed a consistent pattern in altered hematopoietic stem cell properties detectable by increased sensitivity to a chemotherapeutic agent. Sensitivity of CFU-S and CFU-C to 5-FU was maximal approximately 36 hours after endotoxin or cyclophosphamide and 12 hours after irradiation. This increased sensitivity returned

to control levels by 72 hours after endotoxin, but required a longer period of time to recover after cyclophosphamide and irradiation. This latter difference is not surprising in view of the extensive decrease in colony-forming cells seen in marrows of control mice treated with these two cytotoxic agents.

Proliferation of marrow CFU-S after injection of mice with endotoxin has previously been measured either by a 30-minute in vitro exposure to tritiated thymidine (3) or by a 2-hour in vivo exposure to hydroxyurea (11).[8] The former technique gave a clear definition of the kinetics by which CFU-S are stimulated into a state of active proliferation following endotoxin, while the latter demonstrated only marginal differences. The present studies using in vivo 5-FU showed kinetics of sensitivity very similar to those seen using in vitro tritiated thymidine (3), and suggest that changes in sensitivity to 5-FU may also be used to measure the proliferative state of marrow CFU-S. 5-FU blocks DNA synthesis by inhibiting thymidylate synthetase (12) and, like tritiated thymidine (13) or hydroxyurea (14), kills cells in the DNA synthetic (S) phase of the cell cycle (15). However, on the basis of the dose-response curve obtained with 5-FU (1,2) and the possibility that 5-FU may also inhibit RNA synthesis (16,17), 5-FU has been classified as an agent that kills throughout the cell cycle and not only in S phase (1). Such a mechanism for the lethal action of 5-FU, together with the longer exposure period of 6 hours, may account for the hundredfold effect seen in this study compared with the twofold effect seen using in vitro tritiated thymidine (3) or the marginal differences seen using in vivo hydroxyurea (11).

Sensitivity to 5-FU might also be enhanced by factors unrelated to proliferation. One possibility is that 5-FU could interfere with recovery from sublethal damage to nucleic acids resulting from cyclophosphamide or irradiation. Such an explanation has been offered for the increased sensitivity observed when irradiation was immediately followed by in vivo 5-FU (18) or by in vitro tritiated thymidine (19–21). It is unlikely, however, that this would account for a large measure of the sensitivity to 5-FU seen in our studies, since a marked effect was also seen after endotoxin which has not been reported to cause sublethal damage to nucleic acids. It is possible, though, that the very early development of sensitivity to 5-FU seen after irradiation, but not after treatment with endotoxin or cyclophosphamide, could be explained in part by mechanisms that reveal sublethal events.

Regardless of the mechanism of action, a 6-hour exposure to 5-FU, administered at intervals over a clearly defined period of 3–5 days after three different types of marrow perturbation, results in enhanced killing of CFU-S and CFU-C. The technique used in these experiments could thus be adopted as an in vivo screen for detecting and quantitating specific agents that might stimulate hematopoietic stem cells. (In this regard, it is of interest that in preliminary experiments using erythropoietin, cortisone, and serum from heavily irradiated mice, no increased sensitivity of hematopoietic colony-forming cells to 5-FU has been observed.) These results suggest an approach to studying combinations and schedules of chemotherapeutic agents in an effort to minimize marrow toxicity. Since endotoxin is a bacterial cell wall component, these results also suggest caution in administering cytotoxic chemotherapy too soon after an infection or after the use of immunotherapeutic bacterial agents.

[8] Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1.

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Inhibition of Rauscher Leukemia Virus and Avian Myeloblastosis Virus DNA Polymerases by Tilorone (NSC-143969) and its Analogs [1,2,3]

Millie P. Schafer,[4] Michael A. Chirigos,[5] and Takis S. Papas [5,6]

SUMMARY

A potentially chemotherapeutic drug, tilorone, and 14 of its analogs were found to be potent inhibitors of purified RNA-dependent DNA polymerases (RDDP) from the oncogenic tumor viruses, Rauscher leukemia virus (RLV) and avian myeloblastosis virus (AMV). The RLV RDDP exhibited the greater sensitivity to these compounds. The drug concentration required for 50% inhibition of these two DNA polymerases was not only dependent on the enzyme used, but also on the type of analog added to the reaction mixture. Inhibition, however, was found to be primarily due to the planar ring structure common to all of these compounds. The various group substituents on the planar rings only altered the degree of inhibition. Inhibition was also template specific for the templates tested, synthetic hybrids, poly rA·dT_{12-18} and poly rC·dG_{12-18}, and synthetic copolymer, poly (dA–dT). When the poly rC·dG_{12-18} template was used with one of the more potent analogs, no inhibition was observed. This suggests that the compound preferentially interacts with the A–T nucleotide sequences of the various templates.

[Cancer Chemother Rep 58:821–827, 1974]

Tilorone, a broad-spectrum antiviral agent (1) and interferon inducer (2), has shown antitumor activity against the Walker 256 and reticulum cell sarcomas (3). It was later shown that five analogs of tilorone exhibited different degrees of activity against Friend leukemia virus (FLV); however, no correlation was found between the antitumor effects and the drug's interferon-inducing capacity (4). Giron and co-workers also observed a lack of correlation between interferon induction and viral protection by tilorone (5).

Another explanation for the observed antiviral activity could be the inhibition of oncornaviral DNA polymerases. Current evidence indicates that these polymerases play an essential role in the onset of viral expression. Therefore, their inhibition could provide an important tool for suppressing oncogenic information (6,7). It has been reported that tilorone is a potent inhibitor of the mammalian RNA tumor viruses, FLV and Moloney sarcoma virus (MSV) (8). We report here that

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[3] Chemical nomenclature for all compounds mentioned in this paper is included in the List of Compounds at the end of the text.

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tilorone and 14 of its analogs exhibit strong but varying degrees of inhibition of purified Rauscher leukemia virus (RLV) and avian myeloblastosis virus (AMV) DNA polymerases.

The ability to suppress viral activity and/or tumor growth via drug-induced DNA polymerase inhibition could also yield insight into the structural components necessary for antiviral and antitumor activity. Such information could be useful in the development of new synthetic antitumor drugs. Therefore, tilorone and its analogs were evaluated further in order to determine their common, active, structural components involved in inhibition. We also present evidence which indicates a possible molecular mechanism of action for these drugs.

MATERIALS AND METHODS

Reagents.—Unlabeled nucleoside triphosphates and deoxytriphosphates were purchased from P-L Biochemicals. Tritiated nucleoside triphosphates and deoxytriphosphates were obtained from Schwarz-Mann. Specific activities (curies/mmol) were: dTTP, 17.3; dATP, 20.9; dCTP, 30; dGTP, 20; and UTP, 12. Calf thymus DNA was obtained from Worthington Biochemicals Corp. Tilorone analogs were supplied by the Merrell-National Laboratories, Division of Richardson-Merrell, Inc. Except for compounds A-5 and D-3, which were dissolved in 4% DMSO, all tilorone compounds were dissolved in water. At the 4% concentration, DMSO was not found to be inhibitory.

Viruses.—AMV was obtained from chicken plasma of infected chicks and was provided through contract NO1-CP-33291 from the Virus Cancer Program, NCI. Cell debris was removed by centrifugation at 600 × *g* followed by a 30-minute centrifugation at 5000 × *g*. The virus was further concentrated and purified as described by Riman et al (9). RLV and Rous sarcoma virus (RSV) were obtained from the Office of Program Resources and Logistics of the Virus Cancer Program, NCI.

Enzymes.—DNA polymerase from AMV was isolated and purified as described by Kacian et al (10). The purified fraction shows two distinct bands on sodium dodecyl sulfate disk-gel electrophoresis, representing the two nonidentical subunits. Purified enzyme was used for subsequent studies. Assay conditions are those of Kacian et al (10). Purified DNA polymerase from *Escherichia coli* was obtained from Biopolymers Laboratory and was assayed as described by Richardson et al (11). DNA polymerase from *Micrococcus luteus* was purchased from P-L Biochemicals and assayed by the procedure of Bollum (12). DNA polymerase from RLV was purified and assayed by modifying the procedures of Hurwitz et al (13). DNA polymerase from RSV was assayed using the conditions described by Kacian et al (10) for AMV.

RLV DNA Polymerase Assay

A standard reaction mixture, 0.1 ml, contained 100 mM tris-HCl buffer (pH 8.3), 50 mM KCl, 1 mM $MnCl_2$, 4 mM dithiothreitol, 0.125 mM [^{3}H]-TTP (300 cpm/pmol), 0.5 mM dATP, 200 μg of bovine-serum albumin (BSA), 2 μg of the specified template, and 5 μg of purified RLV DNA polymerase. The order of addition was as follows: tris-HCl buffer, KCl, $MnCl_2$, BSA, dithiothreitol, [^{3}H]-TTP, dATP, and template. These were mixed together followed by the addition of the drug. The reaction was initiated by the addition of the enzyme. This order of addition was followed throughout the studies described in this paper. The reaction mixture was incubated at 37°C for 30 minutes and terminated with cold 10% trichloroacetic acid (TCA). The acid-insoluble material was collected on a Whatman filter and washed with 10% TCA. Radioactivity was determined by scintillation counting.

RESULTS

Inhibition of Oncornaviral DNA Polymerases

All tilorone analogs tested exhibited potent inhibition of RLV and AMV DNA polymerase activity (table 1). The RLV DNA polymerase was the most sensitive to these compounds. In contrast, the nonviral *M. luteus* DNA polymerase exhibited considerably less sensitivity to the analogs, requiring a tenfold

TABLE 1.—Effect of tilorone derivatives on viral and *M. luteus* DNA polymerases

Compound*	Drug concentration required for 50% inhibition† (μg/reaction mixture [0.1 ml])			
	Poly (dA–dT)			Poly rA·dT$_{12-18}$
	RLV	AMV	*M. luteus*	RLV
A–1	1.5	4.0	17.0	1.0
A–2	2.0	3.0	2.0	0.4
A–3	2.0	3.0	10.0	2.0
A–4	NT‡	5.0	25.0	>30.0
A–5	—	—	—	5.0
B–1	5.0	17.0	56.0	5.0
C–1	1.5	3.0	8.0	0.8
C–2	9.0	17.0	20.0	3.0
D–1	0.5	6.0	5.0	1.0
D–2	1.0	3.0	7.0	0.7
D–3	—	—	—	1.0
E–1	7.0	18.0	100.0	5.0
F–1	1.0	2.0	3.0	0.5
F–2	1.7	8.0	33.0	2.0
G–1	2.0	7.0	22.0	3.0

*See fig 1 and List of Compounds for structures and chemical nomenclature.

†Each value was obtained from a concentration curve containing a minimum of 7 points.

‡NT = not tested.

increase in some of the drug concentrations to achieve 50% inhibition. The concentrations required for 50% inhibition of the three DNA polymerase activities were dependent on the enzyme used and on the type of tilorone analog added to the reaction mixture.

Template Specificity

Since the RLV DNA polymerase was found to be the most sensitive polymerase to inhibition by tilorone analogs, this enzyme was utilized for further studies. It was of interest to see the effect of the compounds on DNA- and RNA-dependent DNA synthesis using template primers having the same base composition. Both templates exhibited similar responses (table 1). In most cases, the 50% inhibition level for the different compounds utilizing the two templates resulted in no marked difference in amount of inhibitor required to achieve 50% inhibition.

One of the most potent inhibitors, a fluorene derivative, C–1 (fig 1), was selected for further studies utilizing templates of different base composition. The inhibitory effect was dependent on the type of template used in the assay system. No inhibition was observed with the template containing G–C bases, indicating the preference of the inhibitors for A–T bases (table 2).

Minimum Structural Requirements for Inhibition

Two parent compounds were obtained, 2,6-dihydroxyanthraquinone (D-3) and 2,7-dihydroxy-9*H*-fluoren-9-one (A-5) (*see* fig 1). Both were found to inhibit strongly the RLV DNA polymerase (table 1). Inhibition curves were then prepared for each parent compound and two of its derivatives (fig 2 [A and B]). The degree of inhibition exhibited by each parent compound, when compared to two derivatives, was found to be either greater or lesser than the degree of inhibition shown for the derivatives. Group substituents, therefore, can enhance or diminish inhibition of RLV DNA polymerase activity.

Effect of Compound C–1 on Other Polymerases

To determine whether these drugs exhibit specificity of inhibition with respect to other polymerases, C-1 was tested with two bacterial

TABLE 2.—Effect of various templates on the degree of RLV DNA polymerase inhibition

Template*	Inhibition† (%)
Poly rA·dT$_{12-18}$	74
Poly (dA–dT)	67
Activated DNA	87
Poly rC·dG$_{12-18}$	0

*Reaction conditions are described in Materials and Methods.

†0.5 μg of 2,7-bis (4-piperidinobutyryl) fluorene, dihydrochloride (C-1) was employed in each inhibition assay (0.1 ml).

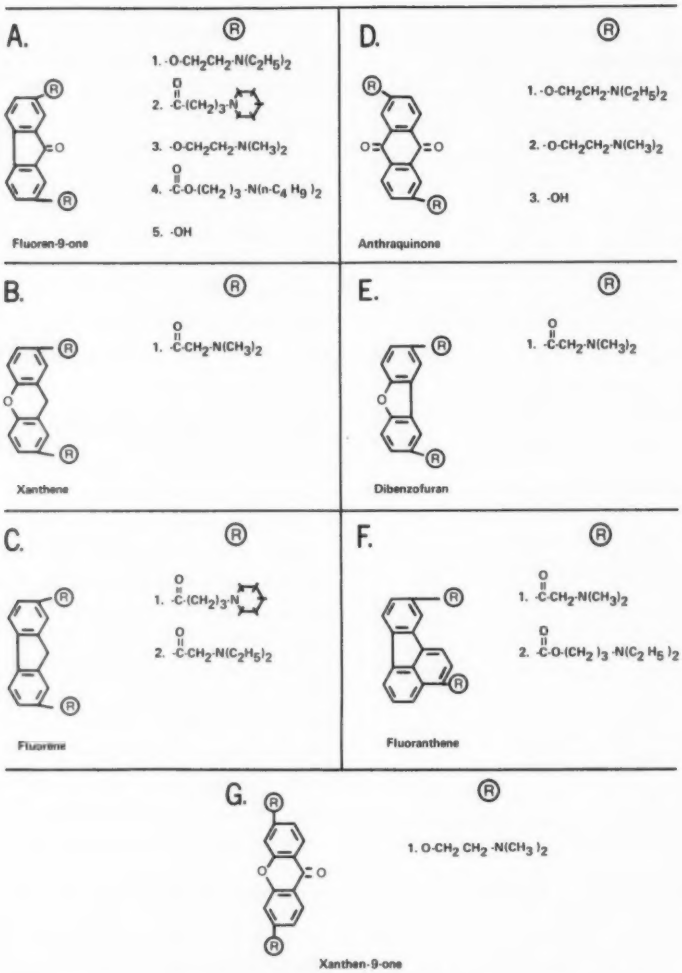

FIGURE 1.—Structures of tilorone (A-1) and its analogs. The two R groups shown on tilorone analogs A–G are substituted by only one of the chemical groups identified by arabic numerals.

polymerases and the RSV DNA polymerase (table 3). Both viral polymerases were inhibited by the drug; the *M. luteus* polymerase was only slightly inhibited. However, the *Escherichia coli* DNA polymerase was mildly activated under the same conditions. These results indicate that the compound shows some specificity for viral polymerases. Studies by Waalkes et al (14) using tilorone also showed that the RLV DNA polymerase was inhibited to a much greater degree than DNA polymerases purified from the Walker 256 carcinosarcoma.

Effect of Concentration of Synthetic Template, poly rA·dT$_{12-18}$, on the Inhibition of DNA Polymerase

To determine the nature of inhibition, template poly rA·dT$_{12-18}$ was varied in the presence and absence of the tilorone inhibitor. The double-reciprocal plots were linear converging lines intersecting below the horizontal

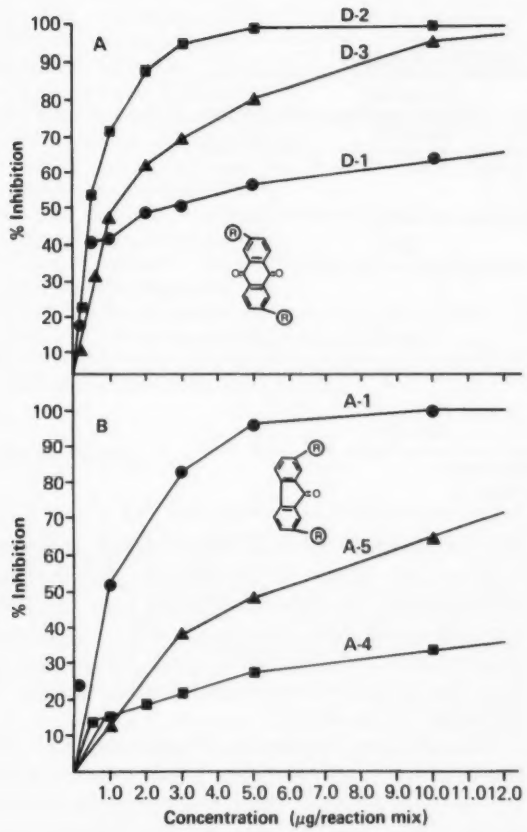

FIGURE 2.—(*A*) Effect of the parent compound, 2,6-dihydroxyanthraquinone (D-3), and two of its analogs on RLV DNA polymerase activity. (*B*) Effect of the parent compound, 2,7-dihydroxy-9*H*-fluoren-9-one (A-5), and two of its analogs on RLV DNA polymerase activity.

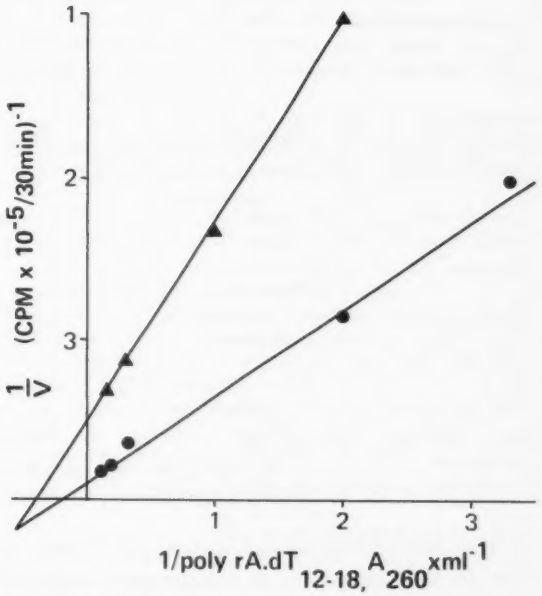

FIGURE 3.—Effect of poly rA·dT$_{12-18}$ template concentration on the inhibition of RLV polymerase. Lineweaver-Burk plot with poly rA·dT$_{12-18}$ as the variable substrate. (●) = No inhibitor; (△) = 0.5 μg/reaction mixture of C-1.

axis (fig 3). The decrease in the reciprocal of the apparent K_m ($-1/K_m$) indicates that the inhibitor altered the binding of the template to the enzyme. A decrease in the maximum velocity (V_{max}) of the reaction was also observed.

DISCUSSION

Seven structural classes of tilorone analogs were used in these studies (fig 1). By varying ring structures and group substituents, information was obtained indicating possible chemical components essential for enzyme inhibition. The most significant common chemical structure is a fused, polycyclic, aromatic ring system which is planar. The strong inhibition exhibited by two parent compounds, D-3 and A-5, would imply that this is the minimum structural requirement for inhibition. Alteration of the middle ring, ie, a five-membered ring versus a six-membered ring, seemed to have little or no effect on the degree of inhibition.

No significant differences in the degree of inhibition were noted between group substituents in the *cis* position, class A compounds for example (fig 1), compared to group substituents in the *trans* position (class D). Surprisingly, the most significant alteration in inhibition was found to be due to group substituents within a particular class. Only one group substituent, the (dibutylamino) propyl group on compound A-4 (fig 1), appeared to diminish strongly inhibition of the RLV DNA polymerase (table 1).

Compounds B-1 and E-1 appeared to be two

TABLE 3.—Effect of 2,7-bis(4-piperidinobutyryl)fluorene, dihydrochloride (C-1) on various polymerases using template, poly (dA-dT)

Enzyme*	Activity in cpm†		
	No drug	C-1 added (0.5 μg/reaction mixture)	Inhibition (%)
RNA-dependent DNA polymerase			
RLV	5991	415	93
RSV	7263	1250	83
DNA-dependent DNA polymerase			
M. luteus	76,796	70,242	9
DNA-dependent RNA polymerase			
E. coli	520,048	625,615	0

*10 μl of enzyme was employed in each assay.
†Each number is the average of 2 values.

of the least effective inhibitors. This may be due to the insertion of an oxygen molecule into the middle ring since the same group substituent, (dimethylamino)acetyl, exhibits strong inhibition when attached to fluoranthene (F-1). Other studies also showed that inhibition of DNA template activity by tilorone and its analogs was strongly influenced by substitutions in the ring (eg, thiophene, furan, etc), as well as in the side chains (15). However, it is clear that group substituents are not essential for inhibition (fig 2); they only alter the degree of inhibition.

Base composition of the synthetic template or DNA seems to be very important in inhibition. At a relatively low concentration of compound C-1, strong inhibition resulted for the poly $rA{\cdot}dT_{12-18}$ and poly (dA–dT) templates. At this same concentration no inhibition was observed when the $rC{\cdot}dG_{12-18}$ template was employed. This would seem to imply that for inhibition to occur, the drug must interact with A–T sequences in the template. The latter result would also indicate that this drug does not exert its effect by inactivating the enzyme. If this were true, the same degree of inhibition would be observed regardless of which template was used.

Chandra et al (8), using synthetic templates, also showed that inhibition of FLV and MSV DNA polymerases by tilorone was dependent on the presence of A–T bases. They later examined the effect of tilorone on the thermal denaturation of DNA and found that the temperature midpoint increased with increasing A–T content of the DNA (16). This implied that the drug preferentially bound to the dA–T portions of the DNA.

The kinetics in figure 3 indicate that the inhibitor-template complex has a greater affinity for the enzyme than the template primer alone. This may be due to the intercalative interaction of the drug with the A–T bases of the template resulting in an enhancement of binding. However, such binding decreases the catalytic activity of the enzyme resulting in a lower V_{max}.

LIST OF COMPOUNDS[7]

A-1: tilorone; NSC-143969; CAS reg. No. 27591-69-1; RMI 10,008; 2,7-bis[2-(diethylamino)ethoxy]-9*H*-fluoren-9-one, dihydrochloride
A-2: RMI 10,556DA; 2,7-bis(4-piperidinobutyryl)-9*H*-fluoren-9-one, dihydrochloride
A-3: RMI 10,635DA; 2,7-bis[2-(dimethylamino)ethoxy]-9*H*-fluoren-9-one, dihydrochloride
A-4: RMI 2557DA; bis[3-(dibutylamino)propyl]-9-oxofluorene-2,7-dicarboxylate, dihydrochloride
A-5: RMI 15,152; 2,7-dihydroxy-9*H*-fluoren-9-one
B-1: RMI 11,513DA; 2,7-bis[2-(dimethylamino)acetyl]-xanthene, dihydrochloride

[7] RMI = Richardson-Merrell, Inc.

C-1: RMI 10,233DA; 2,7-bis(4-piperidinobutyryl)fluorene, dihydrochloride
C-2: RMI 11,002DA; 2,7-bis[2-(diethylamino)acetyl]-fluorene, dihydrochloride
D-1: RMI 10,024DA; 2,6-bis[2-(diethylamino)ethoxy]-anthraquinone, dihydrochloride
D-2: RMI 11,043DA; 2,6-bis[2-(dimethylamino)ethoxy]-anthraquinone, dihydrochloride
D-3: NSC-33531; CAS reg. No. 84-60-6; RMI 10,190; 2,6-dihydroxyanthraquinone
E-1: RMI 11,567DA; 2,8-bis[2-(dimethylamino)acetyl]-dibenzofuran, dihydrochloride
F-1: RMI 11,645; 3,9-bis[2-(dimethylamino)acetyl]-fluoranthene, dihydrochloride
F-2: RMI 9563DA; bis[3-(diethylamino)propyl]fluoranthene-3,9-dicarboxylate, dihydrochloride
G-1: RMI 10,874DA; 3,6-bis[2-(dimethylamino)ethoxy]-9*H*-xanthen-9-one, dihydrochloride

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Antineoplastic and Toxic Effects of *Acinetobacter* and *Pseudomonas* Glutaminase-Asparaginases [1,2,3]

Franz A. Schmid and Joseph Roberts [4,5,6]

SUMMARY

The toxicity of highly purified glutaminase-asparaginases from *Acinetobacter glutaminasificans* (AGA) and *Pseudomonas aureofaciens* (PGA) was studied in C3H/HeJ, CD1, and BDF_1 mice. Daily injections of 250 IU/kg of AGA or 125 IU/kg of PGA in lactate dehydrogenase-elevating virus-infected mice maintained plasma glutamine and asparagine at undetectable levels while glutamate levels rose to 1700–2800 nmol/ml in AGA-treated mice and 3200–4500 nmol/ml in PGA-treated mice. The AGA dose of 250 IU/kg/day × 14 caused weight loss of greater than 3 g only in C3H/HeJ mice; PGA at a dose of 125 IU/kg/day × 14 caused greater than 3-g weight loss in all three strains. In general, such weight loss was accompanied by a decrease in the white blood cell count, platelet count, and spleen weight. There appeared to be an association between the magnitude of the plasma glutamate elevation and the severity of the toxicity. Daily AGA injections of 250–500 IU/kg for 7–14 days resulted in 90%–100% inhibition of total packed cell volume of the following asparaginase-resistant ascites tumors: Ehrlich carcinoma, Taper liver tumor, and Meth A sarcoma. Median survival time was increased up to 100% in AGA-treated mice. At the highest tolerated doses AGA was more effective than PGA. AGA was less effective against solid tumors than against ascites tumors. Combination with 6-diazo-5-oxo-L-norleucine, azaserine, or L-methionine-DL-sulfoximine enhanced the antitumor effects of AGA and PGA. A 75% increase in median survival time was obtained against a subcutaneous myeloid leukemia with a combination of enzyme and analog. Attempts to develop a PGA-resistant tumor failed.

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The rationale for the use of enzymes in the therapy for cancer is based on the principle of decomposing a nutrient in the circulation for which cancer cells have a greater requirement than normal cells (1,2). Tissue culture studies have shown that for certain neoplastic cells glutamine is required at much higher levels than any other amino acid (3,4). Compared with normal tissues, certain tumor cells seem to operate at a marginal level of glutamine availability because of slow synthesis (5) and rapid utilization (6). In 1964, Greenberg et al (7,8) reported that an enzyme preparation from a strain of *Pseudomonas* with glutaminase-asparaginase activities markedly decreased the initial growth rate of several mouse tumors but did not lead to a significant increase in the survival time of the treated animals. Roberts et al (9,10) observed that asparaginase-resistant Ehrlich ascites carcinoma regressed after treatment with highly purified glutaminase-asparaginase. Since that time several reports have been published on the antitumor effect of glutaminase preparations (11–15).

The purpose of the present study was (*a*) to compare the antineoplastic and toxic effects of two different glutaminases in three different strains of mice; (*b*) to study the effect of glutaminase treatment on a spectrum of asparaginase-resistant ascitic and solid tumors; (*c*) to determine the antitumor effect of combinations of glutaminase with L-glutamine antagonists; and (*d*) to attempt to produce a glutaminase-resistant tumor line.

MATERIALS AND METHODS

Animals and Tumors

BDF_1 (C57BL/6 × DBA/2) mice (obtained from A. R. Schmidt Co., Madison, Wis) were the hosts for the following tumors: Taper liver tumor (TLT) in its solid and ascitic form, Ehrlich ascites tumor (EA), E0771 carcinoma (E0771), B16 melanoma (B16), Lewis lung carcinoma (LL), L1210/V leukemia (L1210), L5178Y/CA55 leukemia and its subline L5178Y/CA55/R which was recently made resistant to guinea pig serum asparaginase, and the myeloid C1498 leukemia (C1498). Ridgway osteogenic sarcoma (ROS) was grown in $AKD2F_1/J$ (AKR/J × DBA/2J) mice, EARAD-1 and its subline EARAD-1/R which is resistant to guinea pig serum asparaginase were grown in $B6AF_1/J$ (C57BL/6J × A/J) mice, and Meth A sarcoma was grown in (BALB/cJ × C57BL/6J)F_1/J mice; all three hybirds were purchased from the Jackson Laboratory, Bar Harbor, Maine. For the toxicity studies, C3H/HeJ mice were obtained from the Jackson Laboratory and the CD1 mice from the Charles River Mouse Farms, Inc., Wilmington, Massachusetts. Groups of five or ten female mice, weighing 19–22 g, were used.

The tumors used in this study were either carried by us or obtained from other investigators at our institute. Only C1498 was purchased from the Jackson Laboratory.

For the growth of the ascites tumors, 10^7 cells were injected intraperitoneally (ip), except for L1210 (10^5 cells), EARAD-1 (4×10^5 cells), L5178Y/CA55 (10^6 cells), and in one TLT experiment (10^2 cells). For the development of solid tumors, 10^7 cells were injected subcutaneously (sc) into the right axillary region for TLT and Meth A, 10^6 cells for C1498, and 4×10^5 cells for LL. E0771, B16, and ROS were developed by means of transplantations of small fragments into the right axillary region. The viability of the ascites cells was determined by the dye-exclusion method. Cells were counted in 0.025% trypan blue-0.85% NaCl solution. The ascitic fluids used for tumor implantation contained at least 95% viable cells.

It is known that certain enzymes, eg, asparaginase (16) or *Acinetobacter* glutaminase-asparaginase (14), are cleared at a considerably slower rate from the peripheral blood of mice injected with the lactate dehydrogenase-elevating virus (LDH virus) than in corresponding virus-free mice. Most transplantable mouse tumors have been found to be infected with the LDH virus (17). Therefore, to ensure uniform experimental conditions, we injected ip 0.1 ml of 1:10 diluted plasma from LDH virus-infected mice (17) into all mice, usually 3 days before the start of an experiment.

Enzymes

Acinetobacter glutaminase-asparaginase (AGA) was isolated from *Acinetobacter glutaminasificans* (ATCC 27197). The enzyme was purified to homogeneity as previously described (18). The specific activity of the pure enzyme was 180 ± 20 IU of L-glutaminase and 150 ± 16 IU of L-asparaginase per milligram of protein.

Purified *Pseudomonas* glutaminase-asparaginase (PGA) was supplied as a lyophilized powder by Farbenfabriken Bayer AG (19). The enzyme was isolated from *Pseudomonas aureofaciens* (ATCC 13985) and had a specific activity of 147 IU of L-glutaminase and 104 IU of L-asparaginase per milligram of protein.

Purified *Escherichia coli* asparaginase was obtained from Merck, Sharp and Dohme Laboratories. The specific activity of the enzyme was approximately 300 IU per milligram of protein.

Protein Determination and Enzyme Assays

Protein was determined by the method of Lowry et al (20) using crystalline bovine albumin as the standard. Glutaminase and asparaginase activities were determined by direct nesslerization of the NH_3 produced in their reactions (18). Enzyme and substrate blanks were included in all assays, and a standard curve was prepared with ammonium sulfate. One IU of activity is defined as that amount of enzyme which catalyzes the formation of 1 μmol of ammonia per minute under the conditions of the assay.

Amino Acid Determinations

Amino acid analyses were performed on a Beckman Model 119 amino acid analyzer using lithium citrate buffers for the acidic and neutral amino acids (14,21). Blood for determination of plasma amino acids was obtained from mice by the orbital bleeding technique (14). Special care was taken to minimize amino acid alterations during the processing of blood and plasma samples for amino acid analysis (14).

Glutamine Antagonists

DON and azaserine were supplied by the Drug Development Branch, Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland.[7] L-Methionine-DL-sulfoximine (MSO) was purchased from Sigma Chemical Company, St. Louis, Missouri. All drugs were dissolved in saline and injected ip or sc, usually in a volume of 0.01 ml/g of body weight.

Toxicity Tests

For the toxicity studies, groups of 20 LDH virus-infected CD1, C3H/HeJ, and BDF_1 mice were used. Enzymes were injected ip once daily for 14 days at a dose of 250 IU/kg for AGA and 125 IU/kg for PGA. Twenty control mice of each strain were injected with an equivalent volume of saline. Three mice were selected at random on each day listed 24 hours after the last enzyme injection for determination of various parameters. All blood samples were taken by the orbital bleeding technique. Monocytes and intermediate forms between monocytes and lymphocytes were pooled and included in the lymphocyte count. For the determination of the hematocrit levels, the blood samples were centrifuged at 10,000 rpm for 7 minutes in an International hematocrit centrifuge. Platelet counts were done according to Wintrobe (22) except that the blood was obtained by collecting two drops from a jugular incision on a Na_2EDTA-coated watch glass.

Chemotherapeutic Tests

The day of tumor transplantation was Day 0. The various treatment schedules are indicated later in the text or in the tables.

The effect of treatment on the ascites tumors and leukemias was evaluated on the basis of the average total packed cell volume (TPCV) and/or the median survival time (MST). The difference in the weight of the mouse before tumor inoculation and its weight after removal of ascitic fluid represented the carcass weight change. After the ascitic fluid was centrifuged at 10,000 rpm for 7 minutes in an International hematocrit centrifuge, the TPCV was calculated as the volume of ascitic fluid multi-

[7] DON: NSC-7365; 6-diazo-5-oxo-L-norleucine.
Azaserine: NSC-742; CAS reg. No. 115-02-6; L-serine, diazoacetate (ester).

plied by the fraction of the volume occupied by the packed tumor cells.

The sc implanted tumors were measured in two perpendicular diameters. The average tumor diameters (ATD) were pooled for the various groups of mice.

The effects of treatment were determined on the basis of the ratio of the parameters (MST, TPCV, ATD) of treated tumors (T) to the value of such parameters of corresponding controls (C) (tumor index [T/C]). The most significant parameter in evaluating antitumor efficacy is the MST, however, measurements of TPCV and ATD were used as secondary parameters.

All tumors used, with the exception of L5178Y/CA55 and EARAD-1, were insensitive to *E. coli* asparaginase. Daily ip injections of *E. coli* asparaginase at a dose of 250 IU/kg for 7 days caused less than a 20% increase in MST for L1210, C1498, and LL tumors. The same dose produced less than a 20% decrease in the ATDs of the solid forms or TPCVs of the ascitic forms of TLT, EA, and Meth A (Schmid F, and Roberts J, unpublished results). E0771, B16, and ROS were found to be resistant to asparaginase by Sugiura (23).

An attempt was made to produce a TLT line resistant to PGA by injecting mice with 125 IU/kg/day of PGA for 7 days starting 1 day after tumor implantation. A treated mouse always served as the donor for the control and treated groups of the next generation.

RESULTS

Toxicity Tests

The plasma glutamine levels of untreated C3H/HeJ mice are only about half those of CD1 mice (fig 1). During treatment with AGA or PGA the glutamine levels became undetectable, and after treatment glutamine reappeared sooner in the AGA-treated mice.

PGA caused greater glutamic acid elevation in plasma than AGA (fig 2). Daily injections of 250 IU/kg of AGA increased the glutamate levels to a maximum of 1700 nmol/ml of plasma in CD1 mice and to 2800 nmol/ml of plasma in C3H/HeJ mice. Injections of 125 IU/kg of PGA produced corresponding plasma glutamate levels of 3200 and 4500 nmol/ml. To explore the possible toxicity of such high glutamate levels, C3H/HeJ mice were injected ip daily for 14 days with various doses of sodium glutamate. None of the injections, including doses of 100 mg/mouse, caused any overt toxicity. During a 90-minute iv infusion of 100 mg sodium glutamate/mouse, the plasma glutamate level rose from 73 to 1775 nmol/ml and then dropped to normal by 5.5 hours after infusion.

PGA was more toxic to the CD1, C3H/HeJ, and BDF_1 mice than AGA (table 1). AGA at 250 IU/kg for 14 days caused weight loss of more than 3 g only in C3H/HeJ mice, whereas PGA at half that dose caused weight loss of more than 3 g in all three strains. Mortality rates were in agreement with the weight loss. There were 20 mice in each experimental group; 15 mice were used for blood counts and five were left as potential survivors. No toxicity deaths occurred in the AGA-treated CD1 mice, whereas with half that dose of PGA, all BDF_1

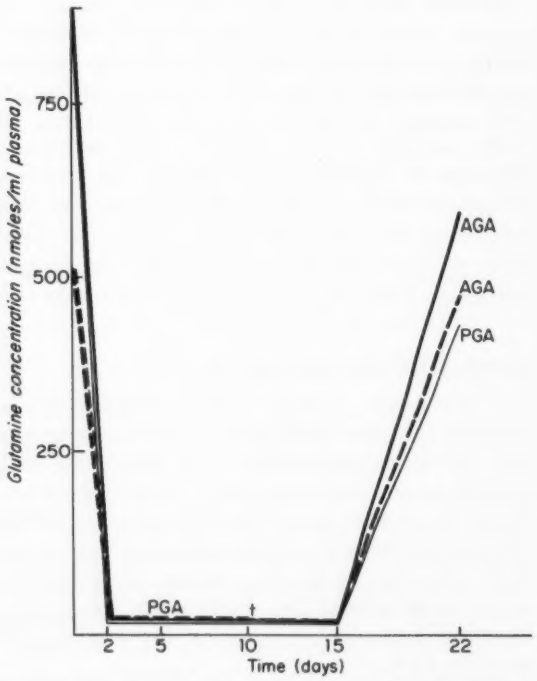

FIGURE 1.—Glutamine levels in the plasma of CD1 (————) and C3H/HeJ (– – – –) mice. AGA and PGA were injected ip once daily for 14 days at doses of 250 and 125 IU/kg respectively.

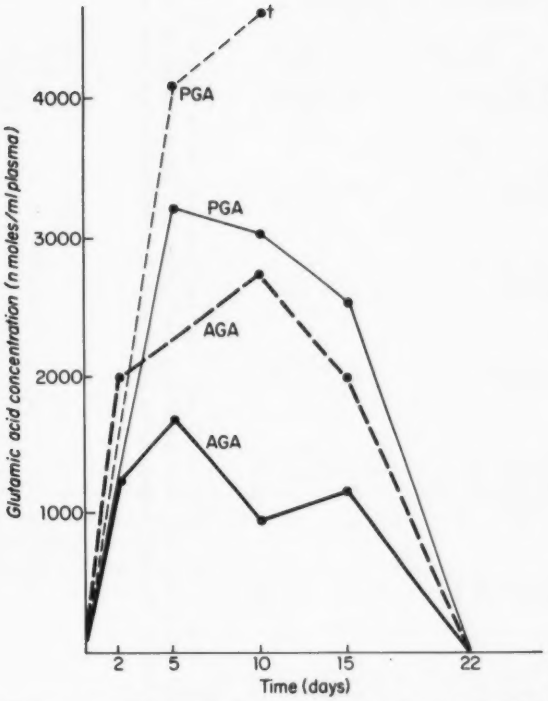

FIGURE 2.—Glutamate levels in the plasma of CD1 (————) and C3H/HeJ (– – – –) mice. AGA and PGA were injected ip once daily for 14 days at doses of 250 and 125 IU/kg respectively.

mice were dead by Day 22 and all C3H/HeJ mice were dead by Day 15. The average spleen weights decreased to less than 50 mg in all PGA-treated groups but only to 80 mg with AGA treatment. Generally, severe body weight loss was accompanied by a decrease in white blood cell (wbc) and platelet counts. The blood counts in the AGA-treated groups showed only a moderate decrease, but treatment with PGA affected blood and platelet counts severely. There appeared to be no differential effect on granulocytes or agranulocytes. The hematocrit values in all treated groups decreased less than 20% from the normal values.

BDF_1 mice tolerated 500 IU/kg of AGA ip for 7 days, and three of five mice survived after treatment with 1000 IU/kg/day. Single doses of up to 6250 IU/kg of AGA were tolerated by these mice with only slight transient weight loss.

Chemotherapeutic Tests

At similar doses AGA and PGA had similar antitumor effects against ascitic TLT as determined by TPCV (table 2). A dose of 250 IU/kg of PGA caused about the same degree of weight loss as 500 IU/kg of AGA. The data in table 3 show results of various dose schedules of AGA treatment on TLT-bearing mice. The greatest increase in lifespan (104%) was obtained with 500 IU/kg for 7 days and 1000 IU/kg for 6 days. When identical treatments were started 9 days after tumor implantation, 500 IU/kg/day caused the greatest increase in lifespan (48%).

The effect of AGA on various ascites tumors is shown in table 4. Daily ip injections of 250 IU/kg for 7 days resulted in 90%–100% inhibition in TPCV of EA, Meth A, and TLT. The percent increase in the MST of corresponding groups was 87, 33, and 27 respectively. The sc administration of enzyme was at least as effective as ip treatment as shown by the TPCVs and MSTs of TLT. Treatment of TLT, delayed until 6 days after tumor implantation, was about as effective as Day 1 treatment, but if treatment was delayed 12 days there was only slight effect, both on TPCV and MST.

Results of tests with AGA against solid tumors are summarized in table 5. The tumors tested were only slightly or not at all inhibited by AGA. Also, intratumoral injections of the enzyme were ineffective, and the formation of lung metastases in LL-bearing mice was not inhibited.

In an attempt to improve the therapeutic effectiveness of AGA on solid tumors, we combined the enzyme with the glutamine-glutamate antagonists DON, azaserine, or MSO. Only a slight inhibition was obtained against the solid form of TLT by the combination of enzyme with DON or azaserine as compared to either antagonist alone (table 6). Each of the combination treatments caused more toxicity, as measured by weight loss, than either drug by itself.

For the combination treatment of the sc implanted leukemia C1498, PGA was used with DON, azaserine, or MSO (table 7). The local tumors were measured at the end of

TABLE 1.—Differential effect of AGA and PGA on 3 mouse strains*

Mouse strain	Day of treatment	AGA (250 IU/kg, Days 1–14) Weight change (g)	AGA Leukocyte count (× 10³/mm³) Total count	AGA Lympho-cytes	AGA Neutro-phils	AGA Platelet count (× 10⁵/mm³)	AGA Spleen weight (mg)	PGA (125 IU/kg, Days 1–14) Weight change (g)	PGA Leukocyte count (× 10³/mm³) Total count	PGA Lympho-cytes	PGA Neutro-phils	PGA Platelet count (× 10⁵/mm³)	PGA Spleen weight (mg)
CD1	Control	+2.0	8.3	6.3	2.0	7.5	164	+2.0	8.3	6.3	2.0	7.5	164
	2	+0.3	6.6	5.6	1.0	4.5	133	+0.7	6.4	5.5	0.9	6.8	163
	5	0	4.0	2.9	1.1	5.4	87	–1.2	5.9	4.9	1.0	6.0	83
	10	+1.3	4.1	3.0	1.1	6.1	126	–5.1	3.3	2.9	0.4	2.0	70
	15	+2.0	7.7	5.9	1.8	5.6	263	–5.0	1.4	1.3	0.1	1.4	38
	22	+3.1	7.6	4.7	3.0	6.1	199	0	6.6	4.1	2.5	6.6	129
	40	5 survivors						1 survivor					
C3H/HeJ	Control	+0.9	8.4	5.6	2.8	6.2	218	+0.9	8.4	5.6	2.8	6.2	218
	2	+0.5	3.1	2.1	1.0	4.8	115	+0.4	6.1	4.3	1.8	5.5	134
	5	–1.3	3.4	2.0	1.4	5.0	85	–2.1	3.0	1.7	1.3	5.5	60
	10	–5.2	4.8	2.3	2.5	5.3	80	–8.7	0.9	0.8	0.1	2.0	48
	15	–5.8	5.1	2.5	3.0	5.3	153	Dead					
	22	–2.3	5.4	2.8	2.6	4.4	191						
	40	No survivors											
BDF_1	Control	+2.0	8.2	6.7	1.5	7.9	106	+1.6	8.2	6.7	1.5	7.9	108
	2	+0.4	6.3	5.3	1.0	7.6	96	–0.7	4.3	3.1	1.2	6.0	101
	5	+0.9	4.1	3.3	0.8	7.9	100	+0.4	4.6	2.8	1.8	3.5	82
	10	–0.2	4.9	2.9	2.0	4.8	149	–2.3	2.3	2.0	0.3	2.3	33
	15	+1.5	6.3	4.4	1.9	5.0	163	–3.0	3.0	2.5	0.5	1.6	45
	22	+1.2	6.3	5.5	0.8	8.9	99	Dead					
	40	3 survivors											

*Three days before treatment the mice were injected with the LDH virus. Groups of 20 female mice were injected daily for 14 days with either AGA (250 IU/kg), PGA (125 IU/kg), or saline. On each day listed 3 randomly selected mice were bled and killed for determination of spleen weight and macroscopic observation. One saline control animal was also killed with each experimental group and these control values were pooled. The values listed are averages.

TABLE 2.—Comparison of effects of AGA and PGA on TLT*

Enzyme	Dose (IU/kg)	Average weight change (g)		TPCV		MST (days)	% ILS‡
		Body	Carcass	MI	T/C†		
AGA	0	+1.9	−2.6	1.70	1.00		
	31	+0.8	−3.1	0.87	0.51		
	62	+0.7	−2.4	0.62	0.37		
	125	−0.3	−1.5	0.19	0.11		
	250	−1.3	−1.4	<0.01	<0.01		
	500	−3.1	−3.2	<0.01	<0.01		
PGA	0	+2.9	−2.5	1.86	1.00		
	31	+0.8	−0.7	0.43	0.23		
	62	+0.9	−0.9	0.39	0.21		
	125	+1.3	+0.6	0.04	0.02		
	250	−3.3	−4.4	<0.01	<0.01		
PGA	0	+0.1				14	
	31	+0.8				15	7
	250	−3.8				30	114

*10^7 cells were injected ip on Day 0; mice received 1 daily ip injection of enzyme from Days 1 to 7. TPCV and weight changes were determined on Day 8. There were 5 BDF_1 mice per test group and 10 controls. No toxicity deaths occurred.

†TPCV of treated mice/TPCV of control mice.

‡ILS = increase in lifespan.

TABLE 3.—Effect of several schedules of administration of AGA on TLT*

Dose (IU/kg/day)	Days of injection	Average weight change (g)		MST† (days)		% ILS‡
		Treated	Control	Treated	Control	
250	1–14	+0.1	+3.8	43	23	87
500	1–7	−2.0	+0.3	47	23	104
1000	1–5	−2.6§	+0.2	47	23	104
3000	1–2	−1.4§	+0.1	42	23	83
5000	1	−0.2	+0.2	40	23	74

*On Day 0 each mouse received 100 tumor cells ip. Enzyme was administered sc. Weight changes were determined 1 day after last injection of the enzyme. There were 5 BDF_1 mice per test group and 20 controls.

†The MSTs of all treated groups differed significantly ($P < 0.01$) from those of the controls.

‡ILS = increase in lifespan.

§One toxicity death occurred in this group.

treatment. PGA alone caused a decrease in tumor size of about 50% and the three glutamine antagonists caused a decrease of less than 20%. The effect of PGA combined with one of the three antagonists, however, was greater than 90%. The increase in lifespan for the three combinations was also slightly more than additive. Markedly increased toxicity was only associated with the MSO-PGA combination. The glutamine antagonists did not interfere with in vivo glutamine breakdown by glutaminase. Plasma amide levels were unaffected by their presence.

An attempt to produce a TLT line resistant to PGA failed. After ten treatment generations with PGA, the TPCV (T/C) values were as follows: control, 1.00; 31 IU/kg, 1.03; 125 IU/kg, 0.13; and 250 IU/kg, 0.04.

TABLE 4.—Effect of AGA and *E. coli* asparaginase on ascites tumors

Enzyme	Tumor	Treatment (IU/kg/day, ip)	Days of injection*	TPCV			MST (days)		% ILS
				Treated	Control	T/C	Treated	Control	
AGA	EA	250	1–7	0.05	1.53	0.03	28	15	87
	Meth A	250	1–7	0.08	1.07	0.07	20	15	33
	L1210	250	1–7	0.16	0.47	0.34	11	9	22
	TLT	250	1–7	0.02	1.88	0.01	19	15	27
	TLT	250	6–12	0.26	3.06	0.08	18	14	29
	TLT	250	12–15	1.37	2.74	0.50	17	16	6
	TLT	250 sc	1–7	0.01	1.80	0.01	24	17	41
	TLT	500 sc	1–7	0.01	1.80	0.01	34	17	100
E. coli asparaginase	TLT	250	1–7	1.59	1.70	0.94			
	TLT	2500	1–7	1.14	1.70	0.67			

*On Day 0 10^7 tumor cells were implanted per mouse except 10^5 for L1210. TPCVs were determined 1 day after last injection of enzyme. There were 5 BDF_1 mice per test group and 10 controls.

TABLE 5.—Effect of AGA on sc tumors

Tumor	Treatment schedule* (days)	Average weight change (g): treated/control (Day 14)	ATD (cm): treated/control		T/C		MST (days)	
			Day 7	Day 14	Day 7	Day 14	Treated	Control
E0771	1–14	+3.6/+1.3	0.78/0.90	1.37/1.68	0.87	0.82	29	24
B16	1–14	+3.6/+0.2	0.60/0.74	1.22/1.55	0.81	0.79	31	25
ROS	1–14	+0.9/+2.3	0.52/0.55	1.20/1.29	0.95	0.93	21	27
Meth A	1–14	+2.7/+0.9	0.48/0.52	0.83/0.88	0.92	0.94	33	22
TLT (10^7 cells)	1–14	+1.7/+1.7	0.61/0.82	1.31/1.42	0.74	0.92		
TLT (10^7 cells)	1–14†	+1.7/+2.6	0.77/0.83	1.36/1.36	0.92	1.00		
LL (4×10^5 cells)	14–27	+0.6/+2.2‡	1.60/1.54§	2.09/2.00‡	1.04§	1.05‡	35	30
LL (4×10^5 cells)	14–27	+2.5/+1.4‡	1.55/1.54§	2.04/2.09‡	1.01§	0.98‡	21¶	19¶

*Tumor was implanted on Day 0. There were 5 or 10 female BDF_1 mice per group. The enzyme dose was 250 IU/kg, ip.
†Intratumoral treatment.
‡Day 28.
§Day 21.
¶No. of lung metastases on Day 28.

The effects of AGA, PGA, and *E. coli* asparaginase on EARAD-1, L5178Y/CA55, and their sublines resistant to asparaginase are presented in table 8. The results obtained with glutaminase against the asparaginase-sensitive and -resistant lines were essentially the same. The increases in survival time ranged only from 5% to 27%. *E. coli* L-asparaginase, in contrast, increased the MSTs of the asparaginase-sensitive lines by more than 300%.

DISCUSSION

Two glutaminases (PGA and AGA) produced by different bacteria caused similar toxicity, but of different intensity, in three mouse strains. C3H/HeJ mice were much more sensitive to the two glutaminases than were CD1 or BDF_1 mice. It is possible that the toxicity of the two glutaminases in the C3H/HeJ mice was due to the greater intracellular depletion of glutamine resulting in

TABLE 6.—Combination treatment of sc TLT*

Treatment (mg/kg)	Average weight change (g): Day 11	ATD		T/C		MST (days)
		Day 11	Day 18	Day 11	Day 18	
Control	+2.1	1.23	1.67	1.00	1.00	46
AGA, 250 IU/kg	+0.6	1.24	1.65	1.01	0.99	50
DON, 0.05	+2.2	1.22	1.59	0.99	0.95	46
DON, 0.1	−0.3	1.06	1.50	0.71	0.90	44
Azaserine, 2.0	+1.8	1.21	1.47	0.98	0.88	50
Azaserine, 4.0	+0.9	1.22	1.47	0.99	0.88	52
AGA + DON, 0.05	−4.9	0.83	1.25†	0.67	0.75	45
AGA + azaserine, 2.0	−2.6	0.93	1.43	0.76	0.86	47

*On Day 0 10^7 tumor cells were injected sc. Treatment was given ip from Days 1 to 10, DON and azaserine in the AM and AGA in the PM. There were 5 female BDF_1 mice per group.

†Two toxicity deaths occurred in this group.

TABLE 7.—Combination treatment of sc transplanted C1498 leukemia

Treatment* (mg/kg)	Average weight change (g): Day 8	ATD (cm): Day 8	T/C	MST ± SE† (days)	% ILS
Control, saline	+0.9	1.51	1.00	12 ± 0.6	
PGA, 125 IU/kg	−0.2	0.70	0.46	15 ± 0.3	25
DON, 0.05	+0.8	1.26	0.83	14 ± 0.5	17
PGA + DON	−1.8	0.08	0.05	19 ± 0.4	58
Azaserine, 2.0	+0.7	1.29	0.85	14 ± 0.6	17
PGA + azaserine	−1.1	0	0	21 ± 2.5	75
MSO, 2.0	+0.8	1.54	1.02	14 ± 0.6	17
PGA + MSO	−3.0	0	0	19 ± 3.3	58

*10^6 tumor cells were injected sc on Day 0. Treatment was given ip from Days 1 to 7, DON, azaserine, and MSO in the AM and PGA in the PM. There were 5 female BDF_1 mice per group and 10 mice in the control and PGA groups.

†All treated groups differed significantly ($P < 0.05$) from the controls.

higher glutamate levels and subsequent cellular injury. PGA was at least twice as toxic as AGA as indicated by the various parameters. Both glutaminases have near optimal activity at physiologic pH and Km values in the 10^{-6} M range. It is possible, however, that molecular differences account for the different toxicities of the two glutaminases. In particular, the substantial difference in the isoelectric points of AGA (18) and PGA (19) may be responsible for differences in the distribution of the two enzymes in the extravascular spaces. The effect on the wbc and platelet counts was similar for both glutaminases and directly related to severe weight loss. Schrek et al (12) tested AGA in vitro against human lymphocytes and found that leukemic lymphocytes were usually killed at the level of 0.0017 IU/ml, whereas the normal lymphocytes resisted the concentration of 1.7 IU/ml.

Results obtained in this study indicate that glutaminase treatment had an inhibitory ef-

TABLE 8.—Comparison of the effects of PGA, AGA, and *E. coli* asparaginase on asparaginase-sensitive and -resistant (R) leukemias*

Treatment (IU/kg)	Average weight change (g): Day 8	MST ± SE† (days)	Average weight change (g): Day 8	MST ± SE† (days)
	EARAD–1		*EARAD–1/R*	
Control, saline	+0.5	15±0.5	+0.6	16±0.5
AGA, 250	−1	19±0.6	−0.5	19±0.6
PGA, 125	−1.5	19±0.7	−1.1	19±0.6
E. coli asparaginase, 250	−4.3	>60	−3.5	17±0.8
	L5178Y/CA55		*L5178Y/CA55/R*	
Control, saline	−0.2	20±0.8	−0.3	20±1.0
AGA, 500	−2.7	23±0.4	−1.8	24±1.3
PGA, 250	−3.7	25±0.9	−5.2	23±0.4
E. coli asparaginase, 500	−4.5	>60	−5.5	21±1.9

*EARAD–1 (4×10^5 cells) and L5178Y/CA55 (10^6 cells) leukemias were injected ip on Day 0. Mice received 1 daily sc injection of enzyme from Days 1 to 14 for EARAD–1 and from Days 1 to 7 for L5178Y/CA55. There were 5 mice per test group and 10 mice for controls.

†The MSTs of all groups, except for the resistant lines which were treated with *E. coli* asparaginase, differed significantly ($P < 0.05$) from those of the corresponding controls.

fect on ascitic tumors and was only slightly inhibitory for solid tumors. It was equally effective by ip or sc administration in preventing the growth of ascites tumors when treatment was started 1 day or 6 days after tumor implantation. Hardy et al (13) reported complete regression of an asparaginase-resistant ascitic methylcholanthrene-induced sarcoma with PGA. Treatment of mice (9,10) bearing the asparaginase-resistant EA tumor with three different glutaminase preparations resulted in complete tumor regression in some animals and prolongation of mean survival time of more than 200%. In general, our results agree with the findings of these investigators. Differences in results may, in part, be attributable to variations in mouse strains, tumor types, and methods employed.

The rationale for using glutamine antagonists in combination with glutaminase is to compensate for the residual glutamine that may be produced by normal cells surrounding the tumor, which could not be depleted by the circulating enzyme. Combinations of glutaminase with DON or azaserine had only slight effect on sc growing TLT but were without effect on the survival of the hosts. A more favorable effect of combination therapy on the increase of host survival time was observed in the treatment of sc myeloid C1498. Using a combination of *Pseudomonas* glutaminase with azaserine, El-Asmar and Greenberg (8) observed a decrease in the number of 6C3HED ascites cells but no significant increase in survival time. Their enzyme was reported to have an unfavorably high Km value (7×10^{-3} M).

Although drug resistance to asparaginase was produced during one treatment generation (24,25), PGA given for ten generations had little effect in selecting or inducing a resistant line. The antitumor effect of asparaginase is believed to be due to the differential content of asparagine synthetase in sensitive and resistant cells; for glutaminase susceptibility, however, such a difference has not been established (15).

Broome and Schenkein (11) observed that 6C3HED sublines resistant to asparaginase appeared to be more strongly inhibited by a PGA

preparation than was the asparaginase-sensitive 6C3HED line. It is known that tumors, while becoming more resistant to certain agents, occasionally become more sensitive, ie, collaterally sensitive, to other agents (26,27). Our tests with the two asparaginase-resistant leukemias EARAD-1/R and L5178Y/CA55/R, however, did not show collateral sensitivity to either of the two glutaminases used here. The relatively poor response of the asparaginase-sensitive leukemias to PGA and AGA which possess both glutaminase and asparaginase activity is also very obvious (18). These tumors are highly sensitive to the effects of *E. coli* asparaginase. These findings are in agreement with those of Riley et al (14) who used AGA against EARAD-1 and of Broome and Schenkein (11) who used a PGA against the asparaginase-sensitive 6C3HED tumor.

On the basis of studies conducted to date, preclinical toxicologic studies are warranted, and we suggest that certain human ascitic tumors and leukemias which are resistant to asparaginase should be responsive to glutaminase therapy.

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Comparison of Postoperative Irradiation Alone and in Combination With BCNU (NSC-409962) in the Management of Malignant Gliomas [1,2]

Steven A. Armentrout, Eldon Foltz, Halvor Vermund, and Peter T. Otis [3,4]

SUMMARY

Eleven patients with malignant gliomas who were treated with postoperative irradiation were compared to 16 patients who were treated with postoperative irradiation and chemotherapy with BCNU. An increased median survival time, 10.0 months compared to 3.0 months, was demonstrated in those patients receiving radiation therapy and chemotherapy. The number of patients alive at 12, 18, and 24 months was also increased in the group treated with BCNU.

[Cancer Chemother Rep 58:841–844, 1974]

The management of malignant gliomas by the combination of surgery, radiation therapy, and chemotherapy has been the subject of much recent interest. The prolongation of survival by radiotherapy remains controversial. There have been frequent reports of lengthy survival rates (1) but at least one report showed no significant life prolongation in a large clinical study (2). A variety of chemotherapeutic agents have been used in the treatment of malignant gliomas either alone or in combination with radiation therapy (3–5). The nitrosoureas have been effective in primary and metastatic malignancies of the central nervous system (6), and effective therapeutic levels of these drugs can be demonstrated in the cerebrospinal fluid after parenteral or oral administration (7).

BCNU has been shown to cause objective improvement and tumor regression in 16 of 30 patients (53%) who have malignant glioma with a mean duration of response of 3.25 months (8). Preliminary reports from a large prospective study suggest that the combination of radiotherapy and BCNU may improve survival in patients with primary malignant gliomas (9).

The purpose of this report is to compare two groups of nonrandomized patients with malignant gliomas who were treated between 1969 and 1973 with surgery and postoperative irradiation, with and without the addition of BCNU chemotherapy.

METHODS

Twenty-seven patients treated for malignant glioma were included in this study. Eleven of these patients were treated with surgery and postoperative irradiation therapy. The other 16 patients were treated with surgery, postoperative irradiation therapy, and intravenous (iv) chemotherapy with BCNU.

The pathologic diagnosis of malignant glioma (glioblastoma multiforme; astrocytoma

[1] Received Jan 7, 1974; revised June 3, 1974; accepted June 21, 1974.

[2] BCNU: CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-. Supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, National Cancer Institute.

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grade III or IV) was confirmed in all patients by our Section of Neuropathology.

The clinical diagnosis of intracranial tumor was established through physical and neurologic examination, brain scan, carotid arteriograms, and in selected instances, pneumoencephalograms. In all patients, craniotomy was performed with a tumor biopsy and as extensive a surgical resection as was possible. One patient in each treatment group had a "second-look" operation with the necrotic tumor being removed during the period of observation.

During the immediate postoperative period, irradiation was administered by a 6-MeV linear accelerator utilizing a technique previously described (1); 5000 rads were administered to the entire brain with an additional 1000 rads being administered to the tumor area for a period of 7 or 8 weeks.

Immediately after radiation therapy, BCNU was administered iv to 16 patients in a dose of 2 mg/kg at 4-week intervals. If the leukocyte count was less than 3000 cells/mm^3 or the thrombocyte count was less than 80,000 cells/mm^3 at 4 weeks, the administration of the drug was delayed for an additional 2 weeks. No significant toxic effects were observed in this series of patients. Informed consent was obtained from all patients who received chemotherapy.

Because of variations in the extent and location of the initial tumor and in the severity of the postoperative deficit, and in view of the poor correlation between a variety of diagnostic tests and tumor regression (10), patient survival was the sole parameter analyzed. Adrenal corticosteroid therapy was used with equal frequency and for equal duration in both groups of patients.

RESULTS

Eleven patients with malignant glioma who were treated with surgery and radiation therapy were compared with the 16 patients who received identical therapy supplemented by chemotherapy with a nitrosourea (BCNU). The median period of observation for the entire patient population was 17 months and the median survival time for the combined patient population was 9.0 months.

Table 1 presents the survival data for all patients in both treatment groups. The median survival time of patients treated with surgery and radiation therapy (3.0 months) differed from that of patients receiving BCNU as additional therapy (10.0 months) (table 2).[5]

In those patients who have been observed for greater than 12 months, one of seven patients (14%) remains alive in the group treated with surgery and radiation whereas six of 11 patients (55%) remain alive among those also receiving BCNU (table 3). After 24 months of observation, one patient treated with surgery and radiation therapy remains alive and three patients who received chemotherapy in addition to surgery and radiation therapy are alive.

Two patients in the series with surgery and radiation therapy survived for 18 months or longer. One patient died at 20 months and the other is alive at 45 months. Four patients are currently alive in the group treated with radiation and BCNU at 18, 24, 24, and 36 months. Four patients are alive among the 11 who received radiation alone with a median survival time between 3 and 7 months. Eleven of the 16 patients receiving BCNU in addition to radiation remain alive with a median survival time of 13 months.

COMMENT

Because of the encouraging reports of the results of chemotherapy in patients with malignant glioma (10), the optimal management of these patients has become the subject of recent intensive investigation. In a recently compiled study of the survival time of 2099 patients with malignant glioma treated with surgery alone (9), the median survival time was 4.5 months with only 10% of the patients being alive at 18 months. The effects of postoperative irradiation on prolonging survival time are controversial and in one study no increase was observed (2). The median survival time for our 11 patients treated with

[5] $\alpha = 0.05 < 0.01$. F test for censored data.

TABLE 1.—Data on 27 patients with malignant glioma treated between 1969 and 1973

Patient No.	Age (yrs)	Sex	Date of therapy (mo/yr)	Survival status*	Survival time (mos)
		Surgery and irradiation			
1	69	F	10/73	A	2
2	53	M	12/69	A	45
3	43	M	5/73	A	7
4	55	M	9/73	A	3
5	67	M	3/69	D	3.5
6	60	M	2/70	D	3
7	70	M	8/73	D	3
8	23	M	9/71	D	2
9	56	M	4/72	D	15
10	31	F	11/71	D	20
11	9	F	3/71	D	18
		Surgery, irradiation, and BCNU			
1	23	M	1/72	A	24
2	30	M	7/72	A	17
3	36	M	1/71	A	36
4	40	M	11/72	A	13
5	60	M	10/72	A†	14
6	33	F	6/73	A	7
7	51	M	11/72	A	13
8	52	M	6/73	A	7
9	69	F	4/73	A	9
10	43	F	1/72	A	24
11	64	M	4/73	A‡	6
12	50	M	5/72	D	8
13	59	F	11/72	D	8
14	35	M	6/72	D	11
15	32	F	1/72	D	9
16	48	M	9/72	D	9

*A = alive; D = dead.
†Lost to followup 11/73.
‡Lost to followup 10/73.

TABLE 2.—Median survival time of patients with malignant glioma

Therapy	No. of patients	Median survival time (mos)	Median length of observation (mos)
Surgery, radiation	11	3.0	26
Surgery, radiation, and BCNU	16	10.0	16

TABLE 3.—Survival time for patients with more than a 12-month followup

Therapy	Total No. of patients	No. of patients—	
		Alive	Dead
Surgery, radiation	7	1	6
Surgery, radiation, and BCNU	11	6	5

postoperative radiation alone (3.0 months) did not differ significantly from the survival reported in previous literature for patients treated with surgery only. Two patients in our group, however, lived for more than 18 months, one patient dying at 20 months and the other still alive at 45 months. Two additional patients survived for periods greater than 12 months (15 and 18 months).

Although the median observation period was shorter for those patients who received chemotherapy, a significant increase in median survival time (10.0 months) was observed. In this group, four patients are alive after 18 months of observation.

Four patients remain alive in the series of 11 patients treated with radiation therapy alone with a median survival time of approximately 4 months. Eleven patients remain alive in the group of 16 patients treated with irradiation and BCNU with a median survival time of 13 months.

Although no attempt at randomization was made in assigning patients to treatment groups, patients were not excluded from either group on the basis of clinical, surgical, or pathologic findings. The surgical techniques and the administration of radiotherapy were identical for both groups of patients, and their supportive medical care as well as their clinical follow-up visits were primarily at a single institution. The difference in median survival time suggests that BCNU administration provides a significant addition to radiation therapy in the management of malignant gliomas. Due to the small sample of patients and relatively short period of observation, however, no definite conclusions can be made. The possibility that the observed differences might be due to factors other than treatment cannot be ignored.

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Comparison of Treatments for Prostatic Cancer Using an Exponential-Type Life Model Relating Survival to Concomitant Information [1,2]

Steven Bayard,[3] Richard Greenberg,[3] Donald Showalter,[3] and David Byar [4,5]

SUMMARY

A multicenter clinical trial of 1824 patients with cancer of the prostate (stages III and IV) randomized into four different treatment groups (estrogen alone [5 mg], orchiectomy plus placebo, orchiectomy plus estrogen [5 mg], and a placebo) is analyzed using an exponential-type survival model with concomitant variables plus other techniques. The agreement of the different methods suggests that the model is useful in determining which factors are more highly associated with mortality and which treatment is preferred when patients exhibit such factors. Specifically, it is suggested that estrogen treatments be given to patients with high acid phosphatase levels but not to older men (75+ years) with signs of cardiovascular impairment. Estrogen is also a high-risk treatment for anemic patients. Other results are given in the text and conclusion.

[Cancer Chemother Rep 58:845–859, 1974]

Models using concomitant patient information have been employed for choosing factors important in affecting a certain outcome variable. Various exponential models of this type using censored survival data are discussed by Breslow (1), who in turn cites additional references. In two recent papers (2,3) a randomized clinical trial conducted by the Veterans' Administration Cooperative Urological Research Group (VACURG) was analyzed using an exponential distribution survival model with the parameter of the distribution described as a linear function of several variables thought to be factors affecting survival in patients with cancer of the prostate. Byar et al (2) used 11 variables and combined 1824 patients who were originally assigned to four different treatments to estimate coefficients and form five risk groups. The authors then found that each of the five observed survival curves was very closely approximated by each of the theoretical predicted survival curves based on a mixture of exponential distributions (the model). The second paper (3) reported on the use of the model with a similar set of ten variables in 302 patients from one of these four treatment groups (placebo) and also showed a good fit. In addition, Greenberg et al (3) used a step-down procedure for eliminating variables in order of their statistical significance with regard to survival. Our paper continues Greenberg's analysis by comparing four different treatments for prostatic cancer: placebo, estrogen alone (5 mg/day orally), orchiectomy

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plus placebo, and orchiectomy plus estrogen (5 mg/day orally). Of special interest is the relative importance of each of the variables to survival for the different treatments. The implication is that no one treatment is best for all patients, but that each treatment has specific actions and side effects; a best treatment exists for an individual with certain symptoms at the time of diagnosis. Thus, we have not combined the patients from the different treatment groups as was done by Byar et al (2) and we have not considered this as a covariance problem with treatment effects since we wish to examine whether the different treatments interact with the concomitant information differently.

Table 1 lists the treatments, the number of patients, and the initial symptoms. The initial symptoms or characteristics we have chosen as concomitant variables for this analysis are age, anemia as determined by hemoglobin level, performance as determined by amount of time confined to bed, metastases [6] of the cancer, and prostatic acid phosphatase level. In addition, the treatments are also categorized by stage of disease advancement [7] at diagnosis, since very real differences in survival rates can be seen for different stages. This is shown in figure 1.

The subset of symptoms was chosen from a much larger subset after extensive preliminary analysis. It is smaller than that used in the studies by Byar et al (2) and Greenberg et al (3) and represents several highly correlated symptoms. For example, a patient with either metastases or an acid phosphatase level greater than 1 King-Armstrong unit at the time of initial diagnosis would usually be classified as stage IV. Nevertheless, it was felt that further information on the effectiveness of treatment could be gained by including all three variables. The other variables included have been analyzed by Byar et al (2) and Mellinger et al (4) and were shown to be related to the force of

[6] These include metastases determined by either roentgenographic or biopsy evidence, either osseous, soft part, or central nervous system involvement.

[7] This paper will deal only with those patients having the two most advanced stages of prostatic cancer, stage IV being the most advanced. A description of these stages, together with a thorough explanation and history of the study, is given in ref 4.

TABLE 1.—Summary of initial symptoms by initial treatment and stage

Treatment	Disease stage	No. of patients					Median acid phosphatase level (% King-Armstrong units)
		Total	Age, 75+ years	Bedridden*	Anemia†	Metastases‡	
Placebo	III	255	51	47	42	2	0.5
	IV	208	23	59	57	87	3.4
Estrogen §	III	253	50	41	40	4	0.5
	IV	202	37	67	61	98	4.1
Orchiectomy + placebo	III	257	55	41	39	0	0.4
	IV	193	29	53	67	94	4.6
Orchiectomy + estrogen §	III	248	59	46	35	2	0.5
	IV	208	34	68	61	108	3.3
All treatments	III	1013	215	175	156	8	0.5
	IV	811	123	247	246	387	3.8
Total		1824	338	422	402	395	0.7

*Those with at least a partial daily confinement to bed at time of diagnosis.
†Those with a hemoglobin level <12 g/100 ml.
‡Shown by roentgenogram or clinical examination.
§ Diethylstilbestrol, 5.0 mg.

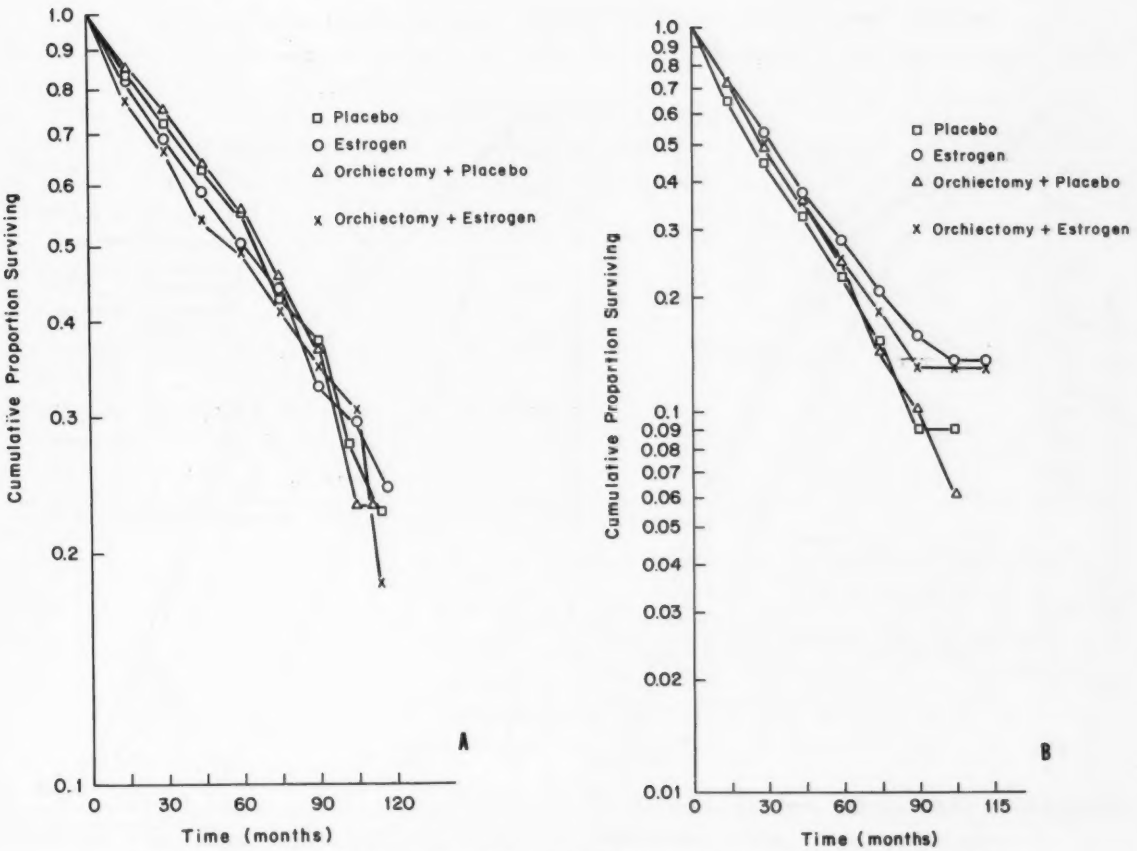

FIGURE 1.—Cumulative survival rates for stage III (*A*) and IV (*B*) cancer patients by initial treatment.

mortality function. In that sense, then, we have preselected what we felt to be important variables affecting survival in prostatic cancer patients and tried to determine which treatments would be best and worst for patients with these signs.

Two factors which complicate the analysis are:

1. More patients initially assigned to receive placebo treatment went off the study than any of the other patient groups as shown in figure 2. We are unable to conclude whether this is because the placebo treatment was ineffective or because the physician simply mistrusted it. Nevertheless, these differential dropout rates must be taken into account. We examined the effect of this differential by analyzing our data as if they were two different data sets. The first analysis determined survival only while the patient was still receiving the initially assigned treatment; the second used all survival information, regardless of the duration of the initial treatment.

2. Patients treated with estrogen have been shown to have better survival rates with respect to cancer, worse survival rates with respect to cardiovascular disease (table 2), and overall survival rates about equal to the other treatment groups. In our analysis we focused mainly on treatment effect for total survival rates but we examined the effect of the estrogen treatments on cardiovascular deaths in older patients.

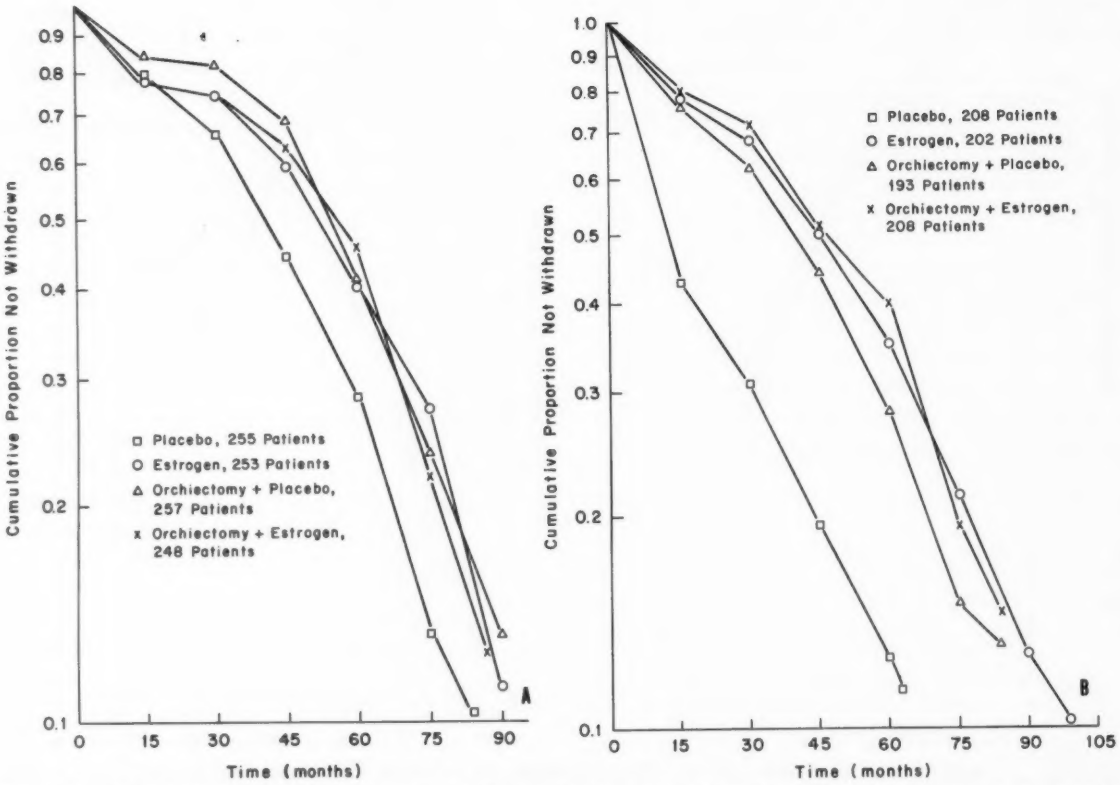

FIGURE 2.—Cumulative nonwithdrawal rates of diagnosed stage III (*A*) and IV (*B*) cancer patients by initial treatment. Withdrawal from the study is defined as switching from the initially assigned treatment or (if that didn't occur) as lost to followup or alive at study cutoff date. Death while still receiving initially assigned treatment is considered a withdrawal for calculation of the nonwithdrawal rates.

AN EXPONENTIAL-TYPE SURVIVAL MODEL USING CONCOMITANT VARIABLES AND TIME DEPENDENCY

The exponential model has been shown to be suitable for analyzing these data (2,3). Probably its best asset is that it enables us to look at all the concomitant variables together in their effect on survival rather than in a series of separate survival rates or cross tabulations. For example, the differential survival rates categorized by stage in figure 1 can be recreated by categorizing by acid phosphatase levels (fig 3). The objective of our analysis is to decide which are the most important variables in the *presence* of others. For this reason each patient has his own force of mortality or intensity function (λ_i) associated with the symptoms he has at the time of his initial diagnosis. Also, this force of mortality is now assumed to be time dependent, since one might not wish to assume that the probability of dying remains constant with time after diagnosis. We call such a model an exponential-type model. Time dependency for the parameter in the exponential model has been discussed by Kodlin.[8] Also, because of the differential withdrawal rates of the various treatments,

[8] Kodlin D. Models of survivorship. Presented at the Epidemiology Association Meeting, Aug 1968.

TABLE 2.—Cause of death by initial treatment and stage: up to 7 years' followup

Treatment	Disease stage	No. of patients dying of— Carcinoma of prostate	Other cancers	Cardiovascular disease*	Other	Total No. of deaths	Total No. of survivors	Total No. of patients studied
Placebo	III	35	9	66	18	128	127	255
	IV	91	3	50	22	166	42	208
Estrogen	III	13	14	85	23	135	118	253
	IV	65	1	66	17	149	53	202
Orchiectomy + placebo	III	30	7	66	25	128	129	257
	IV	79	6	49	19	153	40	193
Orchiectomy + estrogen	III	16	9	86	24	135	113	248
	IV	72	6	51	27	156	52	208
Total		401	55	519	175	1150	674	1824

*Includes all cardiovascular, cerebrovascular, and pulmonary emboli deaths.

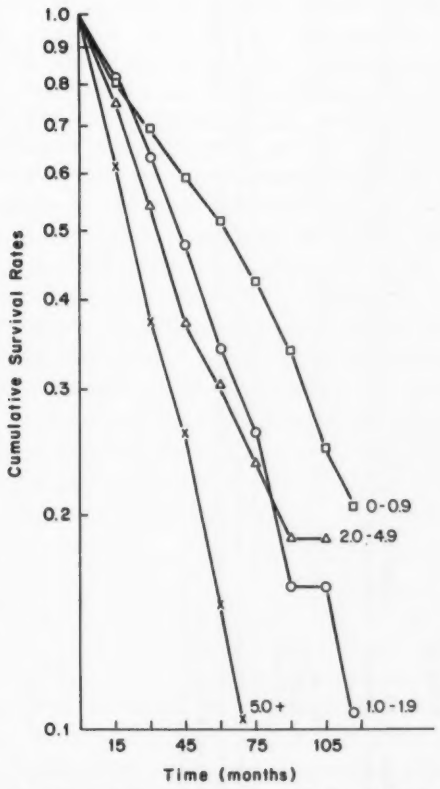

FIGURE 3.—Cumulative survival rates by initial acid phosphatase levels.

time dependency of the force of mortality function allowed a closer examination of the data using either total followup time or time till taken off assigned treatment as the time variable. This time-dependent exponential-type model is discussed in the Appendix.

RESULTS

Total Followup Time Versus Time on Assigned Treatment

The question of how to treat followup patients who change from their originally assigned treatments is constantly arising. Because of the different withdrawal rates for different treatments (fig 2), this question is even more sensitive in our analysis. Therefore, it was decided to analyze the data as two different data sets and compare the results. First, the total time a patient was followed was taken as the time variable (t_α) in the force of mortality (equation A.2, *see* Appendix). This analysis has the heading "total followup time" in table 3. The second analysis substituted the actual time a patient received his assigned treatment; here a patient was considered to be a live withdrawal as soon as he was taken off the treatment to which he was originally assigned whether or not his death was actually recorded subsequently. This analysis has the heading "time on assigned treatment" in table 3. Thus, t_α in equation A.2 takes on different values

TABLE 3.—Estimates of the β_i and their asymptotic standard errors for models with the concomitant variables ("time" used as both "total follow-up time" and "time on assigned treatment")

Treatment			Symptoms: concomitant variables*							
			Mean	Age, 75+ years	Low hemoglobin level (anemia)	Bed confinement	Cancer stage	Metastases	Elevated acid phosphatase level	"Time"
Placebo	Total followup time	Initial set	6.00 ± 1.66	3.77 ± 2.67 (4)	5.02 ± 2.67 (5)	2.95 ± 2.36 (3)	1.85 ± 3.18 (1)	8.96 ± 4.27 (6)†	4.10 ± 1.07 (7)‡	0.0363 ± 0.036 (2)
		Significant subset	8.80 ± 1.11					10.40 ± 4.05	4.64 ± 0.87	
	Time on assigned treatment	Initial set	2.82 ± 2.17	5.15 ± 2.95 (3)	8.68 ± 4.26 (5)†	5.91 ± 3.15 (4)	−4.55 ± 4.18 (2)	30.37 ± 9.14 (6)‡	12.30 ± 2.42 (7)‡	−0.0184 ± 0.040 (1)
Estrogen	Total followup time	Initial set	8.61 ± 1.60	7.72 ± 3.06 (5)†	7.83 ± 3.12 (6)‡	4.42 ± 2.49 (3)	3.08 ± 3.16 (7)‡	5.88 ± 3.58 (4)†	0.93 ± 0.97 (2)	−0.0006 ± 0.035 (1)
		Significant subset	9.60 ± 1.01	7.52 ± 3.06	8.60 ± 3.12		5.43 ± 2.23	7.52 ± 3.53		
	Time on assigned treatment	Initial set	9.10 ± 1.82	9.39 ± 3.69 (4)†	9.76 ± 3.89 (6)‡	7.83 ± 3.32 (5)†	6.20 ± 3.95 (7)‡	6.87 ± 4.59	0.38 ± 1.17	−0.0021 ± 0.042 (2)
Orchiectomy + placebo	Total followup time	Initial set	4.85 ± 1.45	2.37 ± 2.27 (2)	6.79 ± 2.81 (5)†	6.41 ± 2.75 (6)‡	0.63 ± 3.14 (1)	6.14 ± 3.14 (3)	3.45 ± 1.11 (7)‡	0.0664 ± 0.037 (4)
		Significant subset	7.02 ± 1.06		7.02 ± 2.82	7.42 ± 2.75			4.22 ± 0.75	
	Time on assigned treatment	Initial set	5.89 ± 1.67	−0.75 ± 2.17 (2)	6.70 ± 2.97 (5)†	9.35 ± 3.12 (6)‡	1.66 ± 3.48 (3)	6.20 ± 4.64 (4)	3.93 ± 1.27 (7)‡	0.0374 ± 0.042 (1)
Orchiectomy +estrogen	Total followup time	Initial set	9.32 ± 1.72	9.20 ± 3.09 (5)‡	9.54 ± 3.48 (6)‡	4.45 ± 2.56 (3)	1.37 ± 3.01 (1)	11.83 ± 3.77 (7)‡	1.27 ± 1.03 (4) †	0.0248 ± 0.036 (2)
		Significant subset	9.27 ± 1.20	9.66 ± 3.05	10.13 ± 3.46			13.07 ± 3.65	1.61 ± 0.80	
	Time on assigned treatment	Initial set	9.49 ± 2.00	12.20 ± 3.79 (6)‡	9.64 ± 4.14 (5)‡	6.32 ± 3.24 (3)†	−0.06 ± 3.58 (1)	13.60 ± 4.57 (7)‡	1.79 ± 1.19 (4)†	−0.0173 ± 0.049 (2)

* Figures in parentheses indicate the order of elimination of the variables by the step-down procedure by treatment. Estimates of β_i and se ($\hat{\beta}_i$) are from both total set before any elimination by step-down procedure and the subset remaining after elimination of the nonsignificant variables. All estimates are multiplied by 1000 for easier reading.

† Significant at the 0.05 level.

‡ Significant at the 0.01 level.

for those who are withdrawn from treatment. Likewise, n_i in the likelihood equation (A.3) would be different in the two analyses.

Results given in table 3 show little difference in choice between time on assigned treatment and total followup time for our step-down procedure concerning the order with which variables were eliminated from the model. In most cases the order differs by less than one place and nowhere does a vastly different ordering scheme present itself. Also, there are few changes in the attained significance levels of the variables. As far as the time variable is concerned, in no treatment is time statistically significant and it is always among the first variables eliminated by the step-down method. On the other hand, the variances of the estimated coefficients are always smaller when using total followup time, a condition caused by the maximum likelihood estimation procedure. It would thus seem that for these data (*a*) total followup time should be used as the random variable in this model, and (*b*) the force of mortality function need not be complicated by the time-dependent constraint. For the purpose of this analysis, with a maximum total followup of 10 years, the assumption of a constant force of mortality is preferable to one linearly dependent on time.

Before proceeding, however, it is important to mention the changes in the magnitude of the coefficients for the placebo treatment variables in the two analyses, especially those coefficients for the mean, cancer stage, metastases, and acid phosphatase. Since only the coefficients in the placebo group change we suspect that those patients discontinuing the originally assigned placebo treatment differed from those continuing to receive the placebo. Specifically, it appears that the older, anemic, bedridden, metastatic, placebo patients with high acid phosphatase levels died fastest, but when the survivors were switched to other treatments these symptoms had less effect. Also notable is the negative coefficient for cancer stage for the time on assigned treatment. This indicates that those in the most advanced stage had lower mortality till withdrawal, probably because they were withdrawn at a much faster rate. For the remainder of this paper our discussion will pertain to the analysis of total followup time only.

Age

Age at entry in the study was divided into two categories, less than 75 years and at least 75 years. Table 3 displays a fairly clear pattern: neither the placebo nor the orchiectomy treatment groups showed age to be statistically significant, while the estrogen-alone treatment showed significance at the 0.05 level and the orchiectomy plus estrogen treatment showed significance at the 0.01 level. Death in the older men in these two estrogen treatment groups has been linked more to diseases of the cardiovascular system than to cancer of the prostate as shown in table 4.[9]

Section A of table 4 gives the cumulative survival rates by initial age and initial treatment for patients dying of all causes. The eight sets of rates allow comparisons both between treatments within age groups and between ages within treatment groups. As can be seen from the first four rates there is no difference in survival among any of the treatments for the less than 75 age group. For the 75+ group, however, both estrogen treatments show poorer survival than either of the non-estrogen treatments. Also, for the within-treatments-between-ages comparison, the estrogen and non-estrogen treatments show differences. While survival rates for the less than 75 and 75+ groups appear to be the same for the placebo and orchiectomy plus placebo groups, treatment with estrogen shows that older men had worse survival than younger men. These results are consistent with the significant age effects for both estrogen groups shown in table 3.

Section B of table 4 shows the survival rates for patients who died due to cancer of the prostate. In general, the older men have slightly

[9] These survival rates are calculated by the usual life table formulae. Those patients discontinuing their initially assigned treatments were not considered withdrawals and were actually followed for death. Those dying of diseases other than cardiovascular or prostatic cancer-related were considered withdrawals in the interval in which they died.

TABLE 4.—Cumulative survival rates

Months	Patients <75 years				Patients ≥75 years			
	Placebo	Orchiectomy + placebo	Estrogen	Orchiectomy + estrogen	Placebo	Orchiectomy + placebo	Estrogen	Orchiectomy + estrogen
	A. For all causes of death by initial age and initial treatment							
6	0.87	0.91	0.91	0.88	0.88	0.95	0.85	0.80
12	0.80	0.84	0.84	0.82	0.81	0.83	0.70	0.69
24	0.66	0.69	0.71	0.68	0.60	0.64	0.56	0.51
36	0.55	0.58	0.59	0.55	0.48	0.59	0.44	0.42
48	0.48	0.50	0.50	0.48	0.43	0.50	0.33	0.32
60	0.41	0.42	0.44	0.40	0.36	0.36	0.25	0.28
72	0.33	0.34	0.39	0.35	0.28	0.30	0.20	0.25
84	0.25	0.29	0.32	0.28	0.28	0.20	0.20	0.25
	B. For cancer of the prostate deaths by initial age and initial treatment							
6	0.95	0.98	0.98	0.98	0.97	0.99	1.00	0.98
12	0.91	0.94	0.96	0.95	0.97	0.94	0.97	0.93
24	0.81	0.85	0.92	0.86	0.86	0.87	0.91	0.88
36	0.73	0.79	0.85	0.80	0.82	0.87	0.84	0.80
48	0.69	0.74	0.80	0.77	0.75	0.84	0.77	0.73
60	0.66	0.70	0.77	0.74	0.69	0.79	0.77	0.73
72	0.62	0.62	0.73	0.71	0.69	0.79	0.77	0.73
84	0.57	0.56	0.70	0.71	0.69	0.79	0.77	0.73
	C. For cardiovascular disease deaths by initial age and initial treatment							
6	0.94	0.95	0.94	0.93	0.93	0.98	0.88	0.85
12	0.92	0.93	0.90	0.90	0.87	0.91	0.76	0.79
24	0.87	0.86	0.84	0.84	0.75	0.83	0.70	0.64
36	0.82	0.81	0.78	0.78	0.70	0.78	0.60	0.59
48	0.78	0.77	0.70	0.75	0.70	0.68	0.50	0.51
60	0.71	0.73	0.67	0.68	0.70	0.60	0.39	0.48
72	0.64	0.66	0.64	0.62	0.53	0.60	0.31	0.48
84	0.56	0.63	0.58	0.56	0.53	0.60	0.31	0.48
Total No. of patients treated	389	366	368	363	74	84	87	93

better survival than the younger men receiving either placebo or orchiectomy plus placebo. For the two estrogen treatments, both age groups have roughly the same survival. Of additional interest is the comparison within age groups, which shows that the estrogen treatments are slightly better for the younger men than either placebo or orchiectomy plus placebo.

The whole picture changes, however, when we compare survival rates for patients who died due to cardiovascular disease (section C, table 4). Here it becomes clear why we see an age effect for both estrogen treatments in both tables 3 and 4[A]. While survival for placebo and orchiectomy plus placebo is only slightly better in the less than 75-year-old men compared with the older group, the older men for both estrogen groups clearly show the worst cardiovascular survival, with roughly 50% dying from cardiovascular diseases within 4 years.

Acid Phosphatase Levels, Cancer Stage, and Metastases

The variable of acid phosphatase is statistically significant ($P < 0.01$) for both the placebo and orchiectomy patients (table 3). In both cases it is the most significant variable and is the last to be dropped by the step-down procedure. For the two estrogen treatments, however, this is not the case. For the orchiectomy plus estrogen treatment, it is no longer the most significant variable, dropping out fourth, but it is still significant ($P < 0.05$). For the estrogen-alone treatment it drops out either first or second and is not statistically significant.

Inferences from these results might be that estrogen has a very definite effect in lowering acid phosphatase levels by reducing the growth of metastases, especially bone metastases, in the prostatic cancer patients (5), and might be a recommended treatment for patients with very high initial acid phosphatase levels. Complicating this inference, however, is the fact that a high initial acid phosphatase level is associated with both advanced cancer stage and metastases, as seen in table 1. As seen in table 3 the advanced cancer stage of the estrogen-alone patients becomes the most significant variable influencing survival, although cancer stage is not statistically significant for the three other treatments.

In order to investigate whether or not these results were artifacts of the model it was decided to analyze different subsets of the data. The data were recalculated (for total followup time), each time omitting a specific variable. First, the variable for cancer stage was eliminated from the analysis; then, the metastases variable was taken out and the cancer stage variable put back in. The step-down procedure was used for each subset, eliminating the included variables in turn. The results (table 5) show that for all but the estrogen-alone treatment leaving either cancer stage or metastases out of the analysis (*a*) makes little or no difference in the order in which the variables are eliminated by the step-down procedure, and (*b*) has little effect on the coefficients of the remaining variables but reduces the variance of these coefficients.

The estrogen-alone treatment requires further examination. When cancer stage is omitted from the analysis, acid phosphatase becomes the most significant variable, being eliminated last instead of second. This effect is not seen when metastases rather than cancer stage is the variable omitted from the analysis. In this latter case cancer stage again becomes the most significant variable and acid phosphatase again drops out early.

Since interaction between variables in a model like ours could lead to unusual results, it was originally thought that interaction effects might explain the association between acid phosphatase and cancer stage in the estrogen-alone treatment. This, however, proved not to be the case; the only treatment which showed significant interaction between any combination of the three variables (acid phosphatase, metastases, and cancer stage) was the orchiectomy plus estrogen treatment: this was a third-order interaction. There were no significant second-order interactions.

The conclusion would be that for the estrogen-alone treatment, cancer stage in relation to the other variables was the most highly significant variable and that initial acid phosphatase level is far less important; that is, for this one treatment the high initial level appears to be important mainly in that it occurs in most of the patients with stage IV cancer. For the other treatments acid phosphatase appears to be the genuinely significant variable as discussed above.

Additional evidence presented in table 6 shows the clinical status by stage and treatment of patients who survived 6 months after entry in the study. It is shown that fewer of those patients receiving estrogen still had elevated acid phosphatase levels after 6 months than those receiving non-estrogen treatments. Although deaths occurring less than 6 months after the start of treatment and initial values are not adjusted for, this indicates that estrogen controls acid phosphatase levels and thus limits their effects on survival.

The metastases variable shows significance for three of the four treatment groups and is very nearly significant ($P = 0.06$) for orchiectomy also; it is the most significant variable for the orchiectomy plus estrogen treatment. This pattern indicates that no treatment is

TABLE 5.—Estimates of the β_i and their asymptotic standard errors for models with either the concomitant variable for metastases or cancer stage excluded

Treatment	Variable omitted		Symptoms: concomitant variables*							
			Mean	Age, 75+ years	Low hemoglobin level (anemia)	Bed confinement	Cancer stage	Metastases	Elevated acid phosphatase level	Total followup time
Placebo	Cancer stage	$\hat{\beta}_i \times 10^3$	5.97	3.70 (3)	4.90 (4)	2.89 (2)		9.65 (5)†	4.57 (6)‡	0.0369 (1)
		SE $(\hat{\beta}_i) \times 10^3$	1.67	2.67	2.66	2.35		4.02	0.88	0.037
	Metastases	$\hat{\beta}_i \times 10^3$	5.91	3.79 (3)	5.68 (5)	3.32 (2)	4.69 (4)		4.15 (6)‡	0.0357 (1)
		SE $(\hat{\beta}_i) \times 10^3$	1.64	2.67	2.69	2.38	2.96		1.13	0.036
Estrogen	Cancer stage	$\hat{\beta}_i \times 10^3$	8.61	6.82 (4)†	7.50 (5)‡	4.46 (2)		6.63 (3)†	1.61 (6)‡	0.0006 (1)
		SE $(\hat{\beta}_i) \times 10^3$	1.62	3.07	3.10	2.50		3.42	0.68	0.035
	Metastases	$\hat{\beta}_i \times 10^3$	8.40	7.75 (4)†	8.57 (5)‡	5.17 (3)†	4.51 (6)‡		1.16 (2)	0.0002 (1)
		SE $(\hat{\beta}_i) \times 10^3$	1.58	3.07	3.13	2.49	3.08		0.95	0.033
Orchiectomy + placebo	Cancer stage	$\hat{\beta}_i \times 10^3$	4.83	2.36 (1)	6.77 (4)†	6.38 (5)‡		6.36 (2)	3.60 (6)‡	0.0670
		SE $(\hat{\beta}_i) \times 10^3$	1.45	2.27	2.81	2.75		3.82	0.84	0.037
	Metastases	$\hat{\beta}_i \times 10^3$	4.79	2.23 (2)	7.04 (4)†	7.09 (5)‡	2.46 (1)		3.67 (6)‡	0.0633 (3)
		SE $(\hat{\beta}_i) \times 10^3$	1.44	2.25	2.83	2.76	2.97		1.07	0.037
Orchiectomy + estrogen	Cancer stage	$\hat{\beta}_i \times 10^3$	9.28	9.12 (4)‡	9.40 (5)‡	4.52 (2)		12.17 (6)‡	1.60 (3)†	−0.222 (1)
		SE $(\hat{\beta}_i) \times 10^3$	1.73	3.08	3.47	2.56		3.66	0.75	0.037
	Metastases	$\hat{\beta}_i \times 10^3$	9.29	8.92 (4)‡	10.75 (5)‡	5.36 (3)†	4.64 (2)		1.63 (6)‡	−0.0280 (1)
		SE $(\hat{\beta}_i) \times 10^3$	1.72	3.10	3.54	2.62	2.95		0.99	0.036

* Figures in parentheses indicate the order of elimination of the variables by the step-down procedure by treatment. All estimates of the $\hat{\beta}_1$ and the SE (β_i) are from total subset before any elimination by step-down procedure.

† Significant at the 0.05 level at elimination.

‡ Significant at the 0.01 level at elimination.

particularly successful against the effects of metastases with regard to survival.

Hemoglobin Levels and Bed Confinement (Performance Scores)

Hemoglobin levels and the degree of bed confinement can be used as indicators of healthier conditions. Six-month anemia status of patients by stage and treatment is shown in table 6. Lowering the hemoglobin level, thus producing anemia, is common to both estrogen treatments as is the beneficial lowering of acid phosphatase levels. Correspondingly, the hemoglobin variable in table 3 is highly significant for both estrogen treatments ($P < 0.01$) and much less so (but still significant at the 0.05 level for orchiectomy) for the non-estrogen treatments.

Comparing tables 3 and 6, then, we have evidence of two effects:

1. Not only are initial symptoms important in predicting survival, but changing the level of these symptoms by various treatments also changes the value of the initial symptom in predicting survival. More specifically, lowering hemoglobin levels by the estrogen treatments appears to cause higher mortality. This implies that:

2. Estrogen appears to be a poor choice of treatment for patients with anemia.

Performance scores, like hemoglobin, may also be taken as indicative of overall condition. The cross-tabulation of hemoglobin level and performance scores by stage is given in table 7. It is easily seen that in stage III patients there is no association between hemoglobin levels and performance scores ($P > 0.10$ in all cases), while for stage IV patients, in all groups by initially assigned treatment, anemia (low hemoglobin levels) and poor performance are highly associated ($P < 0.005$ in all cases). In addition, not only do a greater proportion of the stage IV patients display anemia (0.303 vs 0.153 for stage III), a greater proportion are also bedridden (0.305 vs 0.173 for stage III).

What table 7 indicates, then, is the close relationship between anemia and bed confinement for stage IV patients, plus both increased anemia and bed confinement in stage IV versus stage III. Even though these two conditions are highly associated, however, a comparison with table 3 shows that when they are considered together in the force of mortality function, anemia shows the higher association with mortality in three of the four groups. Only in the orchiectomy plus placebo group is initial bed confinement significant when the other variables are included. In this group it has a slightly larger coefficient than anemia in the significant subset.

TABLE 6.—Clinical status of patients who survived 6 months after entry into study by stage and assigned treatment*

Treatment	Stage	Percent of patients with—		Total No. evaluated†
		Elevated prostatic acid phosphatase level	Anemia	
Placebo	III	9.1	8.6	186
	IV	78.9	24.2	95
Estrogen	III	0.6	22.6	168
	IV	17.6	27.7	119
Orchiectomy + placebo	III	1.1	9.8	174
	IV	26.0	19.0	127
Orchiectomy + estrogen	III	0.0	26.6	143
	IV	12.3	30.8	130

*See ref 3.

†In addition to patients who had early deaths, those who had a change in treatment before 6 months were not included in this evaluation.

TABLE 7.—Cross tabulation of initial hemoglobin level and bed confinement by treatment and cancer stage (χ^2 values are included)

Treatment	Bed confinement	No. of patients with hemoglobin level— Stage III Normal	Low	Total	Proportion low	Stage IV Normal	Low	Total	Proportion low	χ^2 and P value for stage— III	IV
Placebo	Yes	43	4	47	0.09	34	25	59	0.42	$\chi^2_1 = 2.65$	$\chi^2_1 = 9.28$
	No	170	38	208	0.18	117	32	149	0.21	$P = 0.10$	$P = 0.002$
	Total	213	42	255	0.16	151	57	208	0.27		
	Proportion	0.20	0.10	0.18		0.23	0.44	0.28			
Estrogen	Yes	34	7	41	0.17	35	32	67	0.48	$\chi^2_1 = 0.06$	$\chi^2_1 = 14.67$
	No	179	33	212	0.16	106	29	135	0.21	$P = 0.81$	$P < 0.001$
	Total	213	40	253	0.16	141	61	202	0.30		
	Proportion	0.16	0.18	0.16		0.25	0.52	0.33			
Orchiectomy + placebo	Yes	33	8	41	0.20	23	30	53	0.57	$\chi^2_1 = 0.71$	$\chi^2_1 = 15.45$
	No	185	31	216	0.14	103	37	140	0.26	$P = 0.40$	$P < 0.0001$
	Total	218	39	257	0.15	126	67	193	0.35		
	Proportion	0.15	0.21	0.16		0.18	0.45	0.27			
Orchiectomy + estrogen	Yes	39	7	46	0.15	39	29	68	0.43	$\chi^2_1 = 0.06$	$\chi^2_1 = 8.65$
	No	174	28	202	0.14	108	32	140	0.23	$P = 0.81$	$P = 0.003$
	Total	213	35	248	0.14	147	61	208	0.29		
	Proportion	0.18	0.20	0.19		0.47	0.48	0.33			

Magnitude of the Coefficients and Significance Testing

With the exception of testing whether each of the partial coefficients (β_i) was significantly different from 0, we have not as yet looked at them more closely. Examining them now with respect to magnitude (table 3), all variables with the exception of acid phosphatase and time have the same comparative magnitude since these variables are binary coded. We might estimate, for example, that for placebo the variable of initial metastases (with a coefficient of 8.96×10^{-3}) is roughly three times more important with respect to mortality than the variable for bed confinement, all other variables remaining constant. The same quantitative estimates could not be made between the variables of metastases and acid phosphatase (with a coefficient of 4.10×10^{-3}) without a qualifying statement. Such a statement might be that a patient with an acid phosphatase level of 4.5 King-Armstrong units has roughly twice as high a mortality risk ($4.10 \times 4.5 \times 10^{-3}$ vs 8.96×10^{-3}) as a patient with metastases, all other variables held constant. Eliminating the acid phosphatase and time variables for the present and examining the treatments separately, we find that the coefficient for metastases is the largest coefficient for the placebo and orchiectomy plus estrogen treatments and is still quite large for the other two treatments. For the two estrogen treatments, both age (75+ years) and low hemoglobin level give very high coefficients, either the largest for that treatment or very close to it. This is further evidence of the effect of estrogen treatment on both older and anemic patients. The coefficients for cancer stage are generally low; this reflects that classification of the cancer stage follows from metastases and acid phosphatase measurements. Thus, this variable, combined with the others, does not add much additional information.

Coefficients can also be compared between treatments, if the total subset is used. Because of the large standard errors for the dichotomous variables, no significant differences were found between treatments for any of the variables (age 75+, anemia, bed confinement, cancer stage, and metastases). For the continuous variable of acid phosphatase, however, the coefficient for placebo was significantly higher than that for either the estrogen or orchiectomy plus estrogen treatment, indicating the importance of estrogen in controlling acid phosphatase levels.

It is also important to mention the "mean" variable shown in tables 3 and 5. Its coefficient might be thought of as a measure of importance of all the variables not included in the analysis. It can be compared numerically to all the included variables except acid phosphatase and time, although the standard errors of the coefficients for the "mean" are considerably smaller. This is because, in a sense, everyone has "mean" symptoms, while fewer patients have anemia, for example.

In comparing the coefficients of the means between treatments we see that the magnitude is much larger for the estrogen treatments than for the non-estrogen treatments. Combined with the knowledge of high cardiovascular mortality from these two treatments, we feel that the inclusion of variables such as blood pressure and/or electrocardiogram might have given further direct evidence of the type of patient for whom these treatments are detrimental.

CONCLUSIONS

In analyzing treatment effects for the stage III and IV prostatic cancer patients from the VACURG study, we have used life tables and cross-tabulations in conjunction with our exponential-type model with concomitant variables and a step-down procedure for eliminating variables. We find that our method has the ability to compare the relative effects of treatment on initial symptoms in the presence of others and, therefore, is distinctly superior to either of the former approaches, although to some extent they do complement each other. Our method is, of course, no better than the subset of variables included, but this is true for other methods also. Addressing this conclusion to our subset of variables we can make the following conclusions:

1. Mortality for patients with cancer of the prostate remained fairly constant for our followup period (approximately 9 years).

2. More patients initially diagnosed in stage

III died of diseases other than cancer of the prostate, regardless of treatment, although those receiving estrogen and orchiectomy plus estrogen treatments had fewer cancer of the prostate deaths and more cardiovascular deaths than patients receiving the two other treatments.

3. More patients initially diagnosed in stage IV died of cancer of the prostate than of other diseases for the placebo and orchiectomy plus placebo treatment groups, but the opposite was true for the estrogen and orchiectomy plus estrogen groups.

In looking for treatment effects on initial symptoms we found that:

4. For the estrogen and orchiectomy plus estrogen treatment groups both older age and anemia were highly significant risk factors compared to the other two treatments. Furthermore, the evidence shows that it is the cardiovascular complications in patients with these symptoms that cause death.

5. For the orchiectomy plus placebo treatment group, confinement to bed at the time of initial diagnosis is a significant risk factor.

6. For all treatments a diagnosis of metastases is a significant risk factor.

7. For the placebo and orchiectomy plus placebo treatments, initial diagnosis of an elevated acid phosphatase level was a highly significant risk factor.

8. For the estrogen and orchiectomy plus estrogen treatments, however, elevated acid phosphatase was a much less significant risk factor. This implies that treatment with estrogen has a highly beneficial effect on acid phosphatase but (from statement 4) a detrimental effect on older (75+) men and men with anemia.

APPENDIX

The following is a development of the model used for our analysis. It resembles an exponential model with parameter λ a linear function of several concomitant variables. The extension here deals with time as an additional independent concomitant variable and the different solution of the likelihood equation caused by this time dependency.

If we let

$$F(t) = P \text{ (death by time } t)$$

$$\frac{dF(t)}{dt} = f(t)\,dt = P \text{ (death during the interval } t, t + dt)$$

and $\lambda(t)dt = P$ (death during the interval $t, t+dt$| survival to time t), then, by the well-established hazard function

$$\frac{f(t)dt}{1 - F(t)} = \lambda(t)\,dt.$$

Upon integration we get

$$-\ln[1-F(t)] = \int_0^t \lambda(\tau)d\tau$$

and simple algebra gives the distribution function of life survival times with parameter $\lambda(t)$

$$F(t) = 1-e^{-\int_0^t \lambda(\tau)d\tau} \qquad \textbf{(A.1)}$$

If we now assume $\lambda_\alpha(t)$, the force of mortality for person α, to be a linear combination of concomitant variables and time, we have

$$\lambda_\alpha(t) = \beta_0 S_{0\alpha} + \beta_1 S_{1\alpha} + \beta_2 S_{2\alpha} + \cdots + \beta_k S_{k\alpha} + \gamma t_\alpha = \sum_{i=0}^{k} \beta_i S_{i\alpha} + \gamma t_\alpha \qquad \textbf{(A.2)}$$

where

$$S_{0\alpha} = 1$$

$$S_{i\alpha} \quad \begin{cases} 1 \text{ if patient } \alpha \text{ has qualitative symptom } i \\ 0 \text{ if patient } \alpha \text{ does not have qualitative symptom } i \end{cases}$$

$$0 < i < 6$$

$$S_{6\alpha} = \begin{cases} \text{actual acid phosphatase level if level is} \leq 5 \text{ King-Armstrong units } \% \\ 5 \text{ if actual acid phosphatase level is} > 5 \text{ King-Armstrong units } \% \end{cases}$$

t_α = either "time on assigned treatment" or "total time followed" depending on which analysis was run (*see* text). The indexing of the symptoms is as follows:

S_0 = mean
S_1 = age (75+ years)
S_2 = hemoglobin level (low)
S_3 = performance score (bedridden)
S_4 = cancer stage (IV)
S_5 = metastases (yes)

The actual symptoms used as concomitant variables for this analysis are given in table 1. Acid phosphatase level is the only quantitative symptom; its coding is more fully discussed in reference 3. In addition, t_α = time in study for person α. Making λ a function of time allows a change in the hazard function as the disease progresses.

The likelihood function can now be written

$$L = \prod_{\alpha=1}^{n_1} e^{-\int_0^{t_\alpha} \lambda_\alpha(\tau)d\tau} \lambda_\alpha(t_\alpha) \prod_{\alpha=n_1+1}^{n} e^{-\int_0^{t_\alpha} \lambda_\alpha(\tau)d\tau}$$

$$= \prod_{\alpha=1}^{n} e^{-\int_0^{t_\alpha} \lambda_\alpha(\tau)d\tau} \prod_{\alpha=1}^{n_1} \lambda_\alpha(t_\alpha)$$

and

$$\ln L = \sum_{\alpha=1}^{n_1} \ln \lambda_\alpha(t_\alpha) - \sum_{\alpha=1}^{n} \int_0^{t_\alpha} \lambda_\alpha(\tau)d\tau \qquad \text{(A.3)}$$

where n_1 patients die and $n-n_1$ survive.

Because equation A.2 is a linear function, the integral in equation A.3 is easily calculated.

$$\int_0^{t_\alpha} \lambda_\alpha(\tau)d\tau = \left(\sum_{l=0}^{k} \beta_l S_{l\alpha}\right) t_\alpha + \frac{\gamma t_\alpha^2}{2}$$

We can then derive equations to estimate the β_l's and γ.

In equation A.3 let

$$D_l = \frac{\partial \ln L}{\partial \beta_l} = \sum_{\alpha=1}^{n_1} \frac{S_{l\alpha}}{\sum_{l=0}^{k} \beta_l S_{l\alpha} + \gamma t_\alpha} - \sum_{\alpha=1}^{n} S_{l\alpha} t_\alpha \qquad l=0,\ldots,k$$

$$D_{k+1} = \frac{\partial \ln L}{\partial \gamma} = \sum_{\alpha=1}^{n_1} \frac{t_\alpha}{\sum_{l=0}^{k} \beta_l S_{l\alpha} + \gamma t_\alpha} - \sum_{\alpha=1}^{n} \frac{t_\alpha^2}{2}$$

This gives $K+2$ equation in $K+2$ unknowns; these must be solved simultaneously to obtain the maximum likelihood estimates. The method of solution used is the Newton-Raphson method. It is discussed in reference 3 and will not be further discussed here.

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Phase II Clinical Trial of Isophosphamide (NSC-109724) in Patients With Advanced Breast Cancer [1,2,3]

David L. Ahmann, Harry F. Bisel, and R. G. Hahn [4,5,6]

SUMMARY

Isophosphamide (4 g/m²) administered by 4-hour infusion on Day 1 was contrasted with the combination chemotherapeutic program of 5-fluorouracil, cyclophosphamide, and prednisone, with or without vincristine, in a randomized clinical trial involving 40 patients with advanced breast cancer. Patients were crossed over to alternate treatment upon evidence of progressive disease after the primary treatment program. Four of 20 patients responded to isophosphamide, seven of 11 patients to 5-fluorouracil, cyclophosphamide, and prednisone, and three of nine patients to 5-fluorouracil, cyclophosphamide, and prednisone with vincristine. Utilizing secondary therapy, no patients responded to isophosphamide and one third of the patients responded to combination chemotherapy. Gastrointestinal toxic effects, usually associated with severe bladder dysfunction, were the primary limiting toxic manifestations of isophosphamide therapy. Cystitis occurred in almost all of the patients receiving isophosphamide and in more than half it was considered severe. Platelet-sparing myelosuppression and alopecia occurred in about 70% of the patients; both manifestations were seen in comparable frequency in those patients receiving multiple-drug therapy. Neurologic toxicity was experienced only by patients in the vincristine treatment group.

[Cancer Chemother Rep 58:861–865, 1974]

In a previous communication we reported a phase II clinical trial of methyl-CCNU [7] (1). That evaluation employed a study design in which patients were randomly assigned to the study agent or a chemotherapeutic regimen that used a combination of 5-fluorouracil, cyclophosphamide, and prednisone, with or without vincristine.[8] The design of that particular study seemed appropriate for a phase

[1] Received Mar 19, 1974; accepted June 21, 1974.

[2] Supported in part by contract NO1-CM-12185 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Isophosphamide: CAS reg. No. 3778-73-2; 1,3,2-oxazaphosphorine, 3-(2-chloroethyl)-2-[(2-chloroethyl)-amino]tetrahydro-, 2-oxide; ifosfamide. Compounds used in this study were supplied by Cancer Therapy Evaluation, DCT, NCI.

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[5] The authors wish to thank Drs. Douglass C. Tormey, Paul P. Carbone, and Stephen K. Carter, NCI, for their critique and assistance, and Dr. William Taylor and Mrs. Helen Golenzer, Department of Statistics, Mayo Clinic, for statistical support and randomization procedures.

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[7] Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-.

[8] 5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU.

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-

II clinical drug trial in patients with advanced breast cancer. This present communication reports our experience in evaluating isophosphamide using the previously described study design.

Isophosphamide is an analog of a previously well-studied agent, cyclophosphamide. Clinical trials with isophosphamide were initiated because it has cytostatic superiority to cyclophosphamide in the L1210 leukemia system and in other systems including the Lewis lung carcinoma.[9] Phase I trials were initially conducted by German investigators and were subsequently extended in this country. Treatment with large pulse doses seemed to offer the most therapeutic advantage.[9] Isophosphamide shares with cyclophosphamide many toxicologic characteristics which include nausea, vomiting, cystitis, epilation, and platelet-sparing myelosuppression (2). Tolerable toxicity reported in a phase I trial by Cohen and Mittelman was experienced at a dose of 5 g/m^2 (2).

Clinical activity with isophosphamide has been observed particularly in the lymphomas, small-cell lung carcinomas, ovarian carcinomas, and in chronic lymphatic leukemia where it has shown substantial activity.[9] Since human breast cancer is reasonably sensitive to alkylating agents and particularly to cyclophosphamide (3), a clinical trial evaluating isophosphamide in order to assess its clinical activity in nonrefractory patients with advanced breast cancer was in order.

The alternate therapy utilized in the control arm of this clinical trial included the use of 5-fluorouracil, cyclophosphamide, and prednisone, with or without the addition of vincristine, in the dose regimen described below that had shown a 50% regression rate in previous studies.

MATERIALS AND METHODS

Forty patients were eligible for this study and were entered between May and December 1972. Upon entering the study all patients who were eligible:

a. had readily measurable disease which lent itself to serial quantitation;
b. had not had previous exposure to radiation therapy to the measurable disease;
c. had not had previous exposure to cytotoxic chemotherapy but were consecutive candidates for cytotoxic chemotherapy;
d. were postmenopausal with metastatic, locally inoperable, or recurrent breast cancer;
e. had had pathologic confirmation of their primary disease and hosted no secondary cancers which could have made the origin of the metastatic disease questionable.

Previous additive hormonal therapy had been employed in most of these patients, but no ablative procedures had been performed except oophorectomies in patients who were premenopausal before entry into the study. For the patient to be eligible these oophorectomies must have been performed at least 4 weeks before the study with metastatic disease progression demonstrable before entry on study. Also, if patients had had a previous estrogenic response, there must have been a 4-week period after cessation of the estrogens and tumor progression must have been demonstrable in the interval.

These patients were informed as to the possible toxic effects, limitations, and investigative nature of this study program. The patients had had no previous irradiation therapy to any major marrow-containing area to an extent which would have compromised bone marrow function. They had a hematologic status adequate for vigorous cytotoxic chemotherapy, ie, a white blood cell (wbc) count greater than 4100 cells/mm^3 and a platelet count greater than 130,000 platelets/mm^3 (institutional normals). All patients had a stand-

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19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan.

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-.

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

[9] Friedman MA, and Carter SK. Clinical brochure: *N*-(2-chloroethyl)-*N'*(-2-chloroethyl)-*N'*-*O*-propylene phosphate diamide (Ifosfamide; NSC-109724). Prepared by the NCI, Bethesda, Md, Sept 1970.

ard work-up prior to entry on study (as previously reported [1]) to characterize the extent of their disease, and the studies in this work-up were repeated at 4-week intervals after therapy when patients returned for reevaluation.

Prior to randomization, patients were stratified according to their menstrual category, disease-free interval, and dominant lesions. The distribution of patients (table 1) was quite similar to previously reported studies from this institution with the patients predominantly having visceral dominant disease (75%) and relatively short free intervals.

After stratification, patients were randomly allocated to one of two treatment programs: isophosphamide, 4 g/m² over a 4-hour infusion given intravenously (iv), or a combination program utilizing 5-fluorouracil, 8 mg/kg, and cyclophosphamide, 4 mg/kg, each given by rapid iv infusion on each of 5 successive days. Prednisone was given orally to the latter group at a dose of 30 mg/day for 2 weeks, 20 mg for the next week, and a maintenance dose of 10 mg/day. Vincristine was administered by random distribution to half of the multiple-drug control group at a dose of 1.4 mg/m² iv (not to exceed 2 mg) on Day 1 and on Day 5 of the treatment program. Wbc counts were obtained twice weekly and platelet counts once weekly for 3 weeks after therapy.

Upon reevaluation 1 month after treatment, all patients who exhibited regression or stability of their disease (vide infra) were retreated with the same program at monthly intervals until there was objective evidence of progressive disease. Those patients who had objective regression of their disease after treatment were crossed over to the alternate treatment program.

An objective regression was defined as a measurable reduction in the size of the malignant lesion(s) by at least 50% of the product of the perpendicular diameters. If metastases to the liver were present, a reduction by a factor of one third of the sum of the linear measurements below the subcostal margins must have occurred. If osseous lesions existed, these must have remained static or improved. All patients were considered to have progressive metastatic disease if any new malignant lesions appeared or if previously noted lesions had increased in size by more than 25%.

RESULTS

The regression rates for each of the programs are shown in table 2. Twenty patients received isophosphamide as primary therapy; four of these patients achieved an objective regression, one of which had a duration of greater than 50 weeks with the other three regressions lasting 28, 13, and 8 weeks, respectively. The responses to isophosphamide were seen after the first course of treatment; of six patients receiving isophosphamide as secondary therapy, none achieved the status of objective regression.

TABLE 1.—Distribution of patients by determinants*

Length of postmenstruation (yrs)	Total No. of patients	Dominant disease: Visceral (free interval)†			Osseous (free interval)			Soft tissue (free interval)		
		Simultaneous	1–5 yrs	5+ yrs	Simultaneous	1–5 yrs	5+ yrs	Simultaneous	1–5 yrs	5+ yrs
<1	5/10	3/4	2/3	0/1	0/2	0/0	0/0	0/0	0/0	0/0
1–5	9/15	3/2	4/9	0/0	0/0	0/1	0/0	0/1	2/2	0/0
5–10	0/1	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0
10+	5/14	0/2	3/7	1/1	0/1	0/0	0/0	1/2	0/1	0/0
Totals	19/40	6/8	9/20	1/2	0/3	0/1	0/0	1/3	2/3	0/0
			16/30			0/4			3/6	

* No. of responses/No. of patients studied; includes crossover responses.

† Time from diagnosis of primary cancer to metastasis.

TABLE 2.—Response to isophosphamide and to multiple drugs with or without vincristine

Agents	Order of treatment	
	Primary	Crossover
Isophosphamide	4/20*	0/6
Multiple drugs†		
Alone	7/11	1/9
With vincristine	3/9	4/6

*No. of responses/No. of patients studied.

†5-Fluorouracil, cyclophosphamide, prednisone.

Of the 20 patients receiving the multiple-drug program with or without the addition of vincristine, ten achieved an objective regression (50%) and a mean length of regression of 45 weeks (median 51 weeks) with several patients still having regression on these programs. Crossover treatment with a multiple-drug program after isophosphamide failure has resulted in five of 15 regressions thus far; this is a lesser regression rate (33%) but not statistically significantly so. This decreased regression rate obtained with secondary multiple-drug therapy is consistent with our previous experience (1) as well as with subsequent studies which are currently under way.

The toxic effects encountered utilizing isophosphamide are outlined in table 3. Myelosuppression occurred in two thirds of the patients with relative platelet-sparing effect. The onset of leukopenia occurred 8 days after treatment and lasted an average of 9.6 days. Five patients had wbc counts below 2000 and two patients had counts below 1000 cells/mm^3; no septic deaths occurred. The most troublesome side effect of isophosphamide was extreme bladder irritation experienced by all patients except two; characterized as moderately severe in most of these patients, it resulted in severe dysuria and often frank hematuria. This problem was compounded by the severe nausea and vomiting which accompanied the administration of isophosphamide since any dehydration increased the bladder symptoms. Those patients who were able to increase their fluid intake either by parenteral or by oral means had fewer and less sustained bladder problems. As noted with its parent compound cyclophosphamide, isophosphamide is an excellent epilator as well. Reduction of the treatment dose for those patients receiving subsequent courses of isophosphamide was necessary because of increasing toxicity. After 9 months of therapy, one patient had acute febrile reaction to isophosphamide that necessitated discontinuation of the drug.

TABLE 3.—Toxic effects of treatment programs*

Toxic effects	Isophosphamide	Multiple drugs†	
		Alone	With vincristine
Myelosuppression			
Wbc count 3000‡	17/24§	13/20	12/15
Platelet count 100,000‡	6/24	8/20	7/15
Gastrointestinal upset	22/24	18/20	12/15
Dermatitis	1/24	0/20	0/15
Alopecia	17/24	14/20	13/15
Neuropathy	0/24	0/20	10/15
Cystitis	22/24	0/20	2/15
Phlebitis	0/24	1/20	1/15

*Includes crossover experience.

†5-Fluorouracil, cyclophosphamide, prednisone.

‡Cells/mm^3.

§No. of responses/No. of patients studied.

The toxicity resulting from a multiple-drug program was similar to that previously reported (1) where the most serious side effect was myelosuppression which occurred in 71% of the patients. The onset of myelosuppression occurred on the average at 11 days and lasted a mean of 9 days. Myelosuppression seemed more severe when vincristine was added to the regimen as did the incidence and severity of alopecia. Neurologic toxicity, of course, was confined to the vincristine treatment group and usually resulted in a reduction of the vincristine dose (1 mg on Day 1 and Day 5) in subsequent courses of these drugs. No cumulative toxicity occurred in the multiple-drug programs except for that seen with vincristine.

COMMENT

The antitumor effect of isophosphamide in relation to its toxicity would seem to obviate the need for further study of this compound employing the dose schedule of this study in patients with advanced breast cancer. It has no apparent increased antitumor effect compared to that reported for cyclophosphamide (3) despite increased toxicity. The severe gastrointestinal and bladder toxic effects experienced with isophosphamide using this dose schedule would not make it an attractive agent for the routine management of patients with disseminated breast cancer.

Our additional experience with the multiple-drug program has been reproducible in previous as well as subsequent studies. Whether the addition of vincristine to the regimen of 5-fluorouracil, cyclophosphamide, and prednisone offers any advantages for this dose schedule is now doubtful but should shortly be clearly defined with the additional cumulative experience of current studies. The multiple-drug program we have used is relatively easy to manage and has continued to be utilized as a standard for phase II studies of agents in our patients with advanced breast cancer.

The study design used in this particular phase II evaluation seemed appropriate. Demonstrated again was the constant multiple-drug response that provided a ready assessment of the study agent, isophosphamide, and it provided some relative value for this new drug in our group of patients with advanced breast cancer. In the combination program we described, secondary therapy tended to be less effective than when the same program was used as primary therapy. This experience has also been consistent with previous studies (1). If secondary treatment with a multiple-drug program is consistently proven less effective than primary multi-drug therapy, the study design employed to evaluate new agents in patients with advanced breast cancer may have to be altered, since investigators may be unwilling to enter patients in a study without prior exposure to multiple-drug programs. Secondary therapy with single agents continues to be disappointing and reflects earlier expressed opinions as to the undesirability of evaluating new agents in patients having had previous exposure to cytotoxic chemotherapy (particularly in those patients having had exposure to multiple-drug programs). New agents available for clinical trial must be given a fair assessment in nonrefractory patients without sacrificing optimum patient management. This may become increasingly difficult to accomplish in the future.

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Effect of Nandrolone Phenylpropionate (NSC-23162E) on the Bone Marrow Suppression Caused by Cyclophosphamide (NSC-26271): A Clinical Trial [1,2]

J. Ian Burn [3,4] and W. M. Cooke [3,5]

SUMMARY

A prospective comparative clinical trial was carried out to study the possible protective effect of the anabolic steroid nandrolone phenylpropionate on the bone marrow in patients with advanced malignant disease who were treated with cyclophosphamide. Patients were randomly allocated to one of two groups. One group of patients received cyclophosphamide therapy alone and the other group received cyclophosphamide plus nandrolone phenylpropionate. There was no difference between the two groups in the rates of fall of the hemoglobin levels and the neutrophil counts. There appeared to be some delay in the rate of fall of the lymphocytes in the patients who received nandrolone phenylpropionate. The administration of the anabolic steroid did not effectively prevent the bone marrow depression usually associated with prolonged therapy with cyclophosphamide.

[Cancer Chemother Rep 58: 867–870, 1974]

The therapeutic value of antimitotic agents in the treatment of malignant disease is restricted by the toxic effects on normal tissues, especially those that affect the bone marrow. The margin of safety between an effective dose and a dangerous toxic dose is often very small. The practice of giving an appropriate antidote synchronously, for example folinic acid with the antimetabolite methotrexate,[6] is an unsatisfactory practice that unfavorably influences the response of the tumor to the cytotoxic agent.

Testosterone [7] and its analogs have been credited with both erythropoietic and leukopoietic activity. Erythropoietic activity was observed incidentally during the treatment of senile osteoporosis and was confirmed in a group of patients who had refractory anemias by Martins (1). The beneficial effect of anabolic steroids in anaplastic anemia was reported by Sánchez-Medal et al (2,3) and by Daiber et al (4). Brodsky et al (5) suggested that anabolic steroids appear to protect against the marrow-suppressive effects of cytotoxic drugs, but Kennedy et al (6) reported that

[1] Received Mar 26, 1974; revised June 10, 1974; accepted June 21, 1974.

[2] Nandrolone phenylpropionate: CAS reg. No. 62-90-8; estr-4-en-3-one, 17β-hydroxy-, hydrocinnamate; Durabolin.

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Endoxana.

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[6] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)-methyl]methylamino]benzoyl]-.

[7] Testosterone: NSC-9700; CAS reg. No. 58-22-0.

large doses of the androgenic hormone testosterone enanthate [8] failed to protect against the marrow-suppressive effects of hydroxyurea.[9]

In 1966, Johnston et al (7) reported an investigation in which leukocyte suppression in rats was studied after the administration of the alkylating agent chlorambucil [10] alone and in combination with nandrolone phenylpropionate. There was an appreciable reduction in leukocyte suppression with the administration of nandrolone phenylpropionate, especially in the lymphocyte series.

Investigations of other protective agents such as the thiols have shown these agents to affect tumor cells equally as well as the blood cells, thus negating their beneficial effect (8). Burn and Malaker,[11] however, found no impairment of the therapeutic index [12] in tumor-bearing rats treated with chlorambucil in combination with nandrolone phenylpropionate.

We decided to carry out a clinical trial to test the possible protective effect of nandrolone phenylpropionate in patients with advanced malignant disease treated with cytotoxic chemotherapy.

METHOD

Thirty-nine patients with advanced nonendocrine cancer were included in the study and were randomly allocated to one of two groups. The first group received cyclophosphamide at a dose level of 5 mg/kg of body weight/day × 20 days. The patients were given an initial dose of 100 mg of cyclophosphamide intravenously (iv) and if there was no adverse reaction, the full course was given. The drug was administered iv for the first 10 days and was given orally for the remainder of the course. The second group of patients received the same course of cyclophosphamide plus 25 mg of nandrolone phenylpropionate intramuscularly on alternate days. The first dose of nandrolone phenylpropionate was given the day before starting the course of cyclophosphamide. The patients remained in the hospital during the 20-day treatment period. Twenty-one patients received cyclophosphamide alone and 18 patients received the combined therapy. The distribution of types of cancers was comparable in the two groups (table 1).

The normal hemoglobin value, platelet count, total leukocyte count, and differential neutrophil and lymphocyte counts were determined for each patient before treatment started by taking the mean value of three pretreatment values. During treatment, the hematologic measurements were taken for each patient on alternate days until the total leukocyte count fell below 3000 cells/mm^3 or until the platelet count fell below 100,000 platelets/mm^3. Thereafter, measurements were taken daily. Treatment was discontinued if the side effects of cyclophosphamide became too severe, if the leukocyte count fell below 1500 cells/mm^3, or if the platelet count fell below 75,000 platelets/mm^3.

Treatment was discontinued before the 10th day in three patients and they have not been included in the hematologic analysis. One of these patients had severe gastrointestinal toxicity and the conditions of the other two patients deteriorated rapidly due to progression of their malignant disease but without any evidence of undue toxicity from the therapy. All three of these patients were scheduled to receive nandrolone phenylpropionate. Ten of the patients receiving cyclophosphamide alone

TABLE 1.—Sites of the primary tumors and distribution between the two treatment groups (all patients had either advanced nonresectable primary cancers or metastatic disease)

Tumor site	No. of patients receiving— Cyclophosphamide	Cyclophosphamide plus nandrolone phenylpropionate
Stomach	4	4
Large bowel	11	8
Ovary	3	2
Other	3	4
Total	21	18

[8] Testosterone enanthate: NSC-17591; CAS reg. No. 315-37-7; androst-4-en-3-one, 17β-hydroxy-, heptanoate.

[9] Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1.

[10] Chlorambucil: NSC-3088; CAS reg. No. 305-03-3; butyric acid, 4-[p-[bis(2-chloroethyl)amino]phenyl]-.

[11] Unpublished data, 1974.

[12] Therapeutic index $= \frac{\text{dose causing 50\% mortality rate}}{\text{dose producing 90\% tumor regression}}$.

and nine of the patients receiving combined therapy completed the full 20 days of therapy. Treatment was stopped prematurely in the remainder of the patients because of excessive bone marrow depression. The bone marrow recovered in all of these patients and there were no deaths attributable to the therapy.

RESULTS

The successive hemoglobin, platelet, and leukocyte measurements were estimated as percentages of the mean pretreatment values for each patient. The successive neutrophil and lymphocyte counts were also estimated separately as percentages of the initial normal neutrophil and lymphocyte values. The *mean* percentage on each day was calculated for all of the patients in the two treatment groups. Results have been expressed as a function of the *mean* percentage change and the length of treatment; the two groups were compared accordingly:

Hemoglobin.—In both groups there was a slow but progressive fall in the level of hemoglobin but there was no difference in the rates of fall between the two groups of patients (fig 1).

Platelets.—The platelet counts varied widely from day to day with a marked scatter in the results obtained. It was impossible to make any realistic deductions from the results although it was clear that there was no appreciable fall in either group of patients.

Leukocytes.—The effect on the total white blood cell counts (wbc) is shown in figure 2. The nadir of the fall occurred around 16–18 days after the start of treatment. The slight rise recorded thereafter was accounted for by the smaller number of patients continuing to receive cytotoxic therapy; treatment was terminated in all those with severe leukopenia. In no patient was the depression irreversible after the cytotoxic agent was stopped at the appropriate time. As seen in figure 2, there was no difference between the two groups of patients in the rates of fall of the leukocytes.

The effect of treatment on the neutrophil counts is shown in figure 3. As in the case of the total leukocyte counts, there was no difference in the rates of fall of the circulating

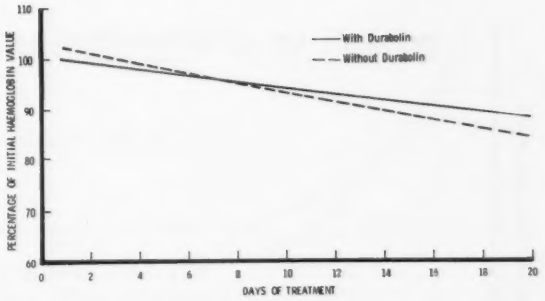

FIGURE 1.—Effect on hemoglobin level. There was no evidence that the co-administration of nandrolone phenylpropionate (Durabolin) prevented the fall in hemoglobin values caused by treatment with cyclophosphamide.

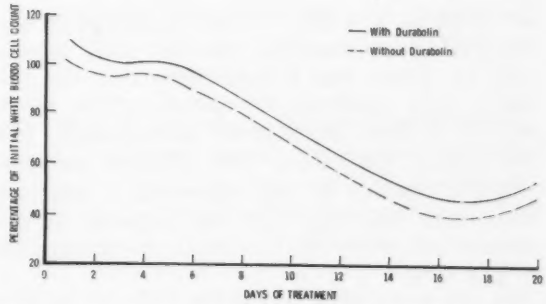

FIGURE 2.—Effect on wbc. The fall in total wbc was not delayed by the co-administration of nandrolone phenylpropionate (Durabolin).

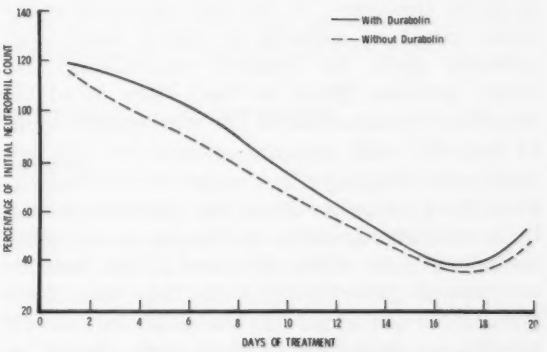

FIGURE 3.—Effect on neutrophil count. Nandrolone phenylpropionate (Durabolin) did not protect the neutrophils from the effects of cyclophosphamide.

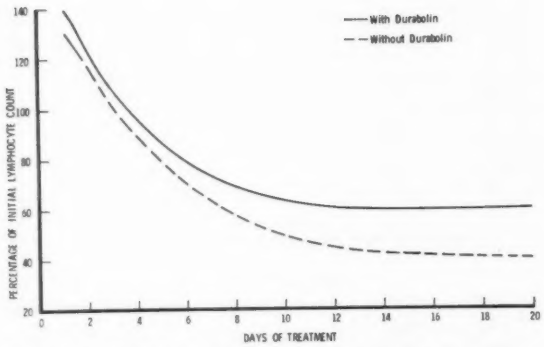

FIGURE 4.—Effect on lymphocyte count. There was a suggestion that the fall in the circulating lymphocyte count caused by treatment with cyclophosphamide was lessened by the co-administration of nandrolone phenylpropionate (Durabolin).

neutrophils. The effect of the treatment on the circulating lymphocytes was less well defined (fig 4). There was a suggestion that the patients who received cyclophosphamide alone showed a more pronounced lymphopenia, but the scatter was rather wide. Because the lymphocyte fraction of the leukocytes is small in man, this difference was not reflected in the rates of fall of the total counts in the two groups of patients.

DISCUSSION

When cyclophosphamide is given at a dose of 5 mg/kg of body weight/day, the depression of the wbc rather than other systemic side effects is usually the factor responsible for stopping the treatment. If the occurrence of leukopenia could be delayed it would mean that patients could be treated continuously for longer periods. Many workers have noted the benefit of testosterone on the hemoglobin count in patients with aplastic anemia but the unfortunate virilizing effect makes its use undesirable. More recently, other less unpleasant anabolic steroids have been shown to have a similar hemopoietic effect. We used a dose level of nandrolone phenylpropionate that was comparable to the dose of the anabolic steroid nandrolone decanoate [13] which was shown by Daiber et al (4) to have a leukopoietic effect.

[13] Nandrolone decanoate: CAS reg. No. 360-70-3.

Our regimen of nandrolone phenylpropionate administration failed to prevent the usual fall in the hemoglobin level and circulating neutrophil count experienced with prolonged therapy with cyclophosphamide. There was perhaps a marginal protection of the lymphocytes which was in accord with the previous findings of Johnston et al (7). Ten of the 21 patients (48%) who received cyclophosphamide alone and nine of the 18 patients (50%) who received cyclophosphamide plus nandrolone phenylpropionate were able to complete the full prescribed course. It is clear, therefore, that in this series of patients the administration of nandrolone phenylpropionate did not beneficially protect the bone marrow from the toxic effects of the cytotoxic agent. In this particular investigation the ability of nandrolone phenylpropionate to facilitate *recovery* of the bone marrow *after* depression was not assessed. In this respect, however, it may be that anabolic steroids will prove to be most useful, and this will be the subject of future study.

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Effects of *Cis*-diamminedichloroplatinum (NSC-119875) on Hearing Function in Man [1,2]

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SUMMARY

Fifty-four serial audiograms performed in 30 patients who had received *cis*-diamminedichloroplatinum were analyzed. Four patients demonstrated significant hearing loss at 2000 Hertz (Hz), 14 at 4000 Hz, and 18 at 8000 Hz. The incidence of ototoxicity increased with increasing doses of the drug. In the presence of renal toxicity, hyperuricemia, or bone marrow depression, decrease of pure-tone threshold at both 4000 and 8000 Hz was more common. Speech-discrimination thresholds showed no consistent pattern of relationships. The possible etiology and pathogenesis of ototoxicity are discussed.

[Cancer Chemother Rep 58:871–875, 1974]

In 1965 Rosenberg and his colleagues (1) observed that inorganic platinum compounds inhibited cell division in *Escherichia coli*. Several neutral platinum-containing chemical species were subsequently shown to have both antibiotic and antitumor activities. *Cis*-diamminedichloroplatinum has had the most extensive trials in transplantable animal tumor systems (2–5) and has been tested in human cancer for several years (6–9).[5] The most commonly reported toxic effects include nausea and vomiting, hyperuricemia, leukopenia, thrombocytopenia, erythroid hypoplasia, and nephrotoxicity (6–9).[5] Audiologic impairment above the frequency range of normal speech has been reported as have occasional single cases of clinical deafness (6–9), but no detailed study has been reported concerning audiologic effects in man. Stadnicki and his colleagues (10) reported *cis*-diamminedichloroplatinum-induced ototoxicity in guinea pigs. Using relatively high doses of the drug, they demonstrated loss of the outer hair cells and occasional loss of the inner hair cells of the cochlea with microscopic evidence of degeneration, cytolysis, and scarring.

MATERIALS AND METHODS

Patients

Audiometric evaluation was attempted in all patients receiving *cis*-diamminedichloroplatinum at Rush-Presbyterian-St. Luke's Medical Center during an 18-month period. All patients had disseminated cancer not treatable by other effective forms of therapy. Thirty patients had serial audiometric evaluation allowing analysis of 54 audiograms which

[1] Received Mar 26, 1974; revised July 13, 1974; accepted July 19, 1974.

[2] *Cis*-diamminedichloroplatinum: CAS reg. No. 1853-33-9; CACP. Supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, National Cancer Institute.

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[5] Hill JM, Speer RJ, Loeb E, et al. Clinical experience with *cis*-platinous diammino dichloride (P.D.D.). Presented at the 7th Congress of Chemotherapy, Prague, Czechoslovakia, 1971.

could be compared to a baseline level. Courses of the drug were administered at doses of 10–100 mg/m²/course either as a single dose or as five daily doses with 3–6 weeks between courses. Audiometric evaluation was usually performed just before a course of therapy. Patients ranged in age from 11 to 62 years, and there were 18 men and 12 women. All patients were closely monitored for hyperuricemia, nephrotoxicity, or depression of bone marrow elements in addition to audiometry.

Audiometric Evaluation

Prior to audiometric evaluation, the ears of all patients were examined for active conductive pathology. The tympanic membrane was viewed and cerumen was removed if necessary. Patients with chronic or acute conduction pathology were eliminated from consideration. No patients complained of hearing difficulty before therapy.

Most testing was performed in a sound-treated test booth (IAC Model No. 402). When the patient was unable to leave his bed or to step up into the test booth, testing was performed in a quiet office or in the hospital room. Ambient noise was reduced as much as possible. Of the 54 hearing tests performed, 49 were obtained using the test booth. All audiologic evaluations were performed by a certified clinical audiologist.

A Beltone 15-C Clinical Audiometer and a Grason-Stadler 1701 Clinical Audiometer were used to obtain thresholds in the test booth. Stimuli were delivered via TDH-39 earphones mounted in MX-41 rubber cushions. Pure-tone thresholds obtained outside the test booth were determined using an Audiotone AU-1 portable audiometer with TDH-39 earphones mounted in MX-41 rubber cushions. All stimuli were calibrated to American National Standards Institute 1969 standards.

Using standard audiometric techniques, all patients were tested in terms of air-conduction pure-tone thresholds. Patients demonstrating decreased thresholds were also tested in terms of bone conduction. Audiometric testing was performed at 250, 500, 1000, 2000, 4000, and 8000 Hertz (Hz) at 5-decibel steps. Speech-reception thresholds and discrimination scores were tested in 22 patients.

RESULTS

Patients were considered to have significant hearing loss if they had at least a 15-decibel decrease in pure-tone threshold in one ear or at least a 10-decibel decrease in both ears as compared to a baseline audiogram. Using these criteria, no patients demonstrated significant hearing loss at any dosage level at frequencies of 250, 500, or 1000 Hz. Three patients had a significant decrease in thresholds at 2000 Hz, 11 at 4000 Hz, and 15 at 8000 Hz.

Table 1 relates hearing loss as defined above to the total dose of *cis*-diamminedichloroplatinum given before audiometric evaluation. These data demonstrate that the greatest incidence of hearing loss occurred at 8000 Hz at doses of 101–200 mg/m²; the least incidence of loss occurred at 2000 Hz at doses of 1–100 mg/m².

Table 2 indicates the degree of hearing loss sustained at each frequency at each dosage level. Hearing loss was bilateral in all but seven audiograms at 8000 Hz and five at 4000 Hz. The greatest degree of hearing loss (50–60 decibels) occurred most often at 8000 Hz, particularly at doses of 201+ mg/m². This degree of loss was noted in only one patient

TABLE 1.—Pure-tone hearing loss vs total dose of *cis*-diamminedichloroplatinum

Dose (mg)	No. of patients examined*	No. of patients with significant hearing loss† at—		
		2000 Hz	4000 Hz	8000 Hz
1–100	18	1	8	6
101–200	15	1	7	11
201+	6	1	2	4

*Some patients examined at more than 1 dose level.
†As compared to baseline audiogram (*see* text).

at 4000 Hz, also at the maximum dosage level. The highest degree of hearing loss at 2000 Hz was only 10–25 decibels at each of the three dosage levels.

Of the 22 patients tested for speech discrimination, 19 had significant pure-tone hearing loss by audiometric testing as defined above. The three patients who did not demonstrate change in pure-tone threshold also had no change in discriminatory threshold. Of the other 19 patients, six demonstrated a decrease in the discriminatory score of 8% or greater, nine showed no change, and four showed improvement in discriminatory ability.

Table 3 demonstrates the relationship between hearing loss and other forms of drug toxicity. Numbers in the table refer to individual measurements after each dose of the

TABLE 2.—Degree of hearing loss vs dose received

Dose (mg)	No. of patients*	Threshold drop of— (decibels)	No. of patients with significant hearing loss†: evaluations performed at— 2000 Hz	4000 Hz	8000 Hz
1–100	18	10–25	1	8	4
		30–45	0	0	1
		50–60	0	0	1
101–200	15	10–25	1	3	7
		30–45	0	4	3
		50–60	0	0	1
201+	6	10–25	1	0	1
		30–45	0	1	1
		50–60	0	1	2

*Some patients examined at more than 1 dose level.
†As compared to baseline audiogram (*see* text).

TABLE 3.—Relationship between audiologic toxicity and other toxicity

Toxic reaction	No. of audiograms showing significant hearing loss*/ No. of audiograms performed in patients with indicated toxic reactions: 4000 Hz		8000 Hz	
Uric acid level				
<6.0 mg/100 ml	7/25		9/24	
>6.0 mg/100 ml	16/29		19/29	
Creatinine level				
<1.5 mg/100 ml	14/40		17/39	
>1.5 mg/100 ml	9/14		11/14	
Hemoglobin level				
<2 g/100 ml decrease	13/33		13/33	
2–3 g/100 ml decrease	4/11	8/17	6/10	11/16
>3 g/100 ml decrease	4/6		5/6	
Platelet count				
>100,000 cells/mm³	19/47		24/47	
<100,000 cells/mm³	4/7		5/6	
White blood cell count				
>5000 cells/mm³	2/8		4/8	
<5000 cells/mm³	5/10		5/10	

*As compared to previous audiogram (*see* text).

drug. Although all patients received allopurinol[6] during the period of drug administration, 18 demonstrated transient hyperuricemia prior to 29 audiograms. Twelve patients had renal toxicity before 14 audiometric evaluations. Of these, nine demonstrated hearing loss at 4000 Hz and ten at 8000 Hz.

Patients who had any evidence of bleeding were not included in the evaluation of anemia, yet 14 patients demonstrated a significant decrease in hemoglobin level. Only 14 patients did not receive a chemotherapeutic agent other than *cis*-diamminedichloroplatinum which might have depressed leukocyte formation. Eight of these patients demonstrated leukopenia.

With regard to each parameter, significant toxicity appears to be related to an increased incidence of ototoxicity. The converse, however, cannot be stated, because six audiograms taken when there had been no other toxicity demonstrated hearing loss.

In order to determine whether the baseline threshold might be related to subsequent ototoxicity, thresholds were compared to those of the previous audiogram in each patient for each ear. Results are shown in table 4. There appears to be no relationship between prior hearing acuity and the probability of having further hearing loss except that patients who already demonstrated significantly depressed thresholds tended not to have further hearing loss.

[6] Allopurinol: NSC-1390; CAS reg. No. 315-30-3; 1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol.

TABLE 4.—Pure-tone hearing loss vs prior acuity

Prior acuity (decibels)	No. of evaluations showing > 10-decibel change/No. of evaluations performed at—	
	4000 Hz	8000 Hz
0	10/45	6/14
5–10	5/17	5/11
15–20	4/6	6/15
25–30	2/10	7/19
35–40	2/7	6/17
45–50	2/10	4/6
55–60	1/8	2/9
65+	2/5	3/14

DISCUSSION

The definition of significant hearing loss applied herein was purposely designed to be conservative. Wertheimer has demonstrated that repeated testing of individuals over a period of 23 successive days resulted in a sigma of thresholds of 1.22 decibels (11). Also, since the testing was performed in 5-decibel steps, a change of two steps in each ear or three steps in one ear was required to qualify as a significant hearing loss.

Even by conservative criteria, *cis*-diamminedichloroplatinum is a potentially ototoxic compound in man, regardless of whether other forms of toxicity are manifested in the patient. In terms of incidence and degree of hearing loss, the drug affects hearing acuity at 2000 Hz only slightly. However, 4000 Hz appears to be a frequency more sensitive even at minimum dosage levels and 8000 Hz appears to be the most sensitive frequency. Although the highest incidence of hearing loss at these two frequencies fell into the 10–25-decibel category, the number of patients sustaining a decrease in threshold beyond this level was greater at 8000 Hz than at 4000 Hz. The incidence of hearing loss appeared to increase at cumulative doses greater than 100 but less than 200 mg/m^2. Two patients complained of tinnitus, both of whom had significant hearing loss at 8000 Hz. No patients complained of noticeable change in hearing function. Patients were not followed sufficiently to determine reversibility of ototoxicity.

No patient had symptoms or findings of vestibular damage. Also, bone conduction, when tested, always corresponded closely to air conduction. These facts are best explained by a pathologic process in the cochlea, consistent with the results of animal experiments by Stadnicki et al (10) referred to earlier. The pathogenesis of these pathologic findings must remain speculative. Studies by Yassin et al have correlated serum electrolyte changes in renal failure with hearing loss (12). It is possible that ototoxicity may be related to serum electrolyte changes secondary (*a*) to direct renal effects of the drug, (*b*) to vomiting and resultant fluid shifts, or (*c*) to generalized cytotoxicity causing changes in mem-

brane permeability and therefore anion shifts. Serum electrolyte levels were not measured in patients reported in this paper, but in future studies these will be closely monitored.

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Controlled Evaluation of Adriamycin (NSC-123127) in Patients With Disseminated Breast Cancer [1,2,3]

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SUMMARY

Forty-eight ambulatory patients with disseminated breast cancer were evaluated in a prospective randomized trial that compared adriamycin (60 mg/m²) with a multiple-drug program employing 5-fluorouracil (5-FU), cyclophosphamide, and prednisone with or without the addition of vincristine. If initial treatment failed, patients were crossed over to the alternate treatment program. Ten of 20 patients responded to primary therapy with adriamycin as compared to eight of 16 who responded to the combination program of 5-FU, cyclophosphamide, and prednisone without vincristine and five of 12 who responded to the combination program with vincristine. Crossover treatment resulted in one response to adriamycin among nine patients treated and seven responses to the multiple-drug programs among 15 patients treated. Toxicity included mild gastrointestinal side effects in 75% of the patients receiving adriamycin and 85% of the patients receiving multiple-drug programs. Leukocyte depression occurred in 70% of the patients receiving adriamycin and in 75% of the patients receiving the multiple-drug programs. The platelet count was suppressed in only one of 20 patients treated with adriamycin and in 20% of the patients receiving the multiple-drug programs. Two patients receiving the adriamycin program had incipient or early congestive heart failure at doses less than 400 mg/m² and the drug was stopped, although the relationship to adriamycin was not proven. Neither of the two patients died and the congestive failure responded to conventional management. Alopecia occurred in all patients receiving adriamycin and was present in 90% of the group receiving the multiple-drug programs. Neurologic toxicity was limited to the group treated with vincristine and required reduction of the vincristine dose in subsequent treatment courses.

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[2] Supported in part by contract N01-CM-12185 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Adriamycin and all other drugs used in this study were supplied by Cancer Therapy Evaluation, DCT, NCI.

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[5] We thank Drs. Douglass C. Tormey, Paul P. Carbone, and Stephen K. Carter, NCI, for their critique and assistance. We also thank Dr. William Taylor and Mrs. Helen Golenzier, Department of Statistics, Mayo Clinic, for statistical support and randomization procedures.

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This study reports the clinical evaluation of adriamycin in the treatment of patients with disseminated cancer of the breast. The study design used in this evaluation was essentially the same as that previously reported (1) in which the new agent, in this case adriamycin, was compared in a randomized trial to a known effective multiple-drug program using 5-fluorouracil (5-FU), cyclophosphamide, and prednisone with or without the addition of vincristine.[7] The purpose of the control arm in this study was to ensure an adequate clinical trial and not to directly compare the efficacy of adriamycin to multiple-drug programs.

Adriamycin is an antibiotic structurally related to daunorubicin;[8] however, its therapeutic index appeared to be higher than that of daunorubicin (2). Adriamycin's mechanism of action seems to be by interference with nucleic acid synthesis of both DNA and RNA (3). Cells in mitosis and in early S phase seem to be the most sensitive to adriamycin action in animal systems. An excellent cell-kill effect was noted at relatively low doses which suggests that the clinical use of a low-dose schedule might further improve the therapeutic index of this drug (3). However, O'Bryan et al reported a dose level of 45 mg/m² as being less effective than one of 75 mg/m² in good-risk patients (4).

An excellent earlier clinical review that outlined preliminary phase I and II experiences by Bonadonna et al (2) indicated that adriamycin may well be more therapeutically active at lower doses than daunorubicin and is effective in a larger variety of neoplastic diseases. A recently published extended phase II clinical trial reported by O'Bryan et al (5) supported the earlier prediction of adriamycin's relatively broad-spectrum effect on a variety of tumors. Specifically, in patients with disseminated breast cancer, a 32% response rate was noted in patients who had failed to respond to primary chemotherapy with conventional drugs.

The toxic effects of adriamycin have included nausea, vomiting, alopecia, stomatitis, myelosuppression, and cardiotoxicity.[9] Adriamycin's unique cardiotoxic effect was not frequently observed, although with total doses that exceed 550 mg/m² the incidence rises and may be manifested by nonreversible cardiac failure (6).

In other studies, the dose schedules for adriamycin have varied. We chose the more promising schedule, one which had resulted in appreciable antitumor activity (7): intermittent single injections at a modest dose of 60 mg/m² every 3–4 weeks.

The alternate treatment program employed in this study used a combination of 5-FU, cyclophosphamide, and prednisone with or without the addition of vincristine. This regimen has previously been reported and has been consistently associated with a 50% response rate in a similar group of patients (1).

MATERIALS AND METHODS

The 48 patients who entered this study were consecutive candidates for consideration for cytotoxic chemotherapy. The patients had known metastatic disease without question of primary origin and without a secondary primary which might cast some doubt on the origin of the metastatic disease. The metastatic disease present was quantitatable, lending itself to serial measurements. The patients were postmenopausal or, if premenopausal, had experienced progressive disease by at least 6 weeks after oophorectomy. Many of the patients had received previous hormonal therapy but their tumors had progressed before they were entered into this study. No patient had received previous cytotoxic chemotherapy and none of the chosen indicator lesions was exposed to previous or concomitant irradiation therapy. All of the patients had adequate mar-

[7] 5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU.

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan.

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-.

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

[8] Daunorubicin: NSC-83142; CAS reg. No. 23541-50-6; daunomycin (NSC-82151).

[9] Blum RH, Friedman MA, and Carter SK. Clinical brochure: adriamycin (NSC-123127), Mar 1972. Prepared by the DCT, NCI, Bethesda, Md.

row functions, ie, white blood cell counts (wbc) over 4100 cells/mm³ and platelet counts exceeding 130,000 platelets/mm³ (institutional normals), and had not received radiation therapy to an extent that might compromise fairly vigorous cytotoxic chemotherapy. A full discussion was held with the patient as to the toxic effects, the limitations of the chemotherapeutic program, and the investigational aspects of the treatment prior to entry into the study.

The pretreatment evaluation was the same as previously reported (1) which is fairly standard for phase II studies. Also included, however, was the addition of an electrocardiogram prior to entry into the study as well as before every anticipated dose of adriamycin. None of the patients had abnormal renal function (serum creatinine value >1.5 mg/100 ml) nor did any have serum bilirubin levels of greater than 2 mg/100 ml. No evidence of active cardiac disease existed in any of these patients.

Before the randomized assignment to a study program, the patients were classified according to the time interval from discovery of the primary lesion to the discovery of metastasis, the interval since last menstruation, and the dominant disease present (table 1).

Patients were assigned at random to one of two treatment programs. Twenty patients received adriamycin at a dose level of 60 mg/m² by intravenous (iv) infusion on Day 1 and repeated every 3–4 weeks; most of the patients were treated at 4 weeks since several initial patients did not recover from myelosuppression within 3 weeks. Sixteen patients received the multiple-drug program which consisted of 5-FU, cyclophosphamide, and prednisone without vincristine, and 12 patients received the multiple-drug program with the addition of vincristine. 5-FU was given at a dose level of 300 mg/m²/day, and cyclophosphamide was given at a dose level of 150 mg/m²/day. Both drugs were given by direct iv infusion on each of 5 successive days. Prednisone was given orally at a dose level of 30 mg/day for 2 weeks, 20 mg/day for 1 week, and a maintenance dose of 10 mg/day. Vincristine was given at a dose level of 1.4 mg/m² (not to exceed 2 mg) on Days 1 and 5.

As the treatment proceeded, doses were adjusted in each patient to produce adequate but

TABLE 1.—Distribution of patients and responses*

Discriminant	Adriamycin	5-FU, cyclophosphamide, and prednisone	5-FU, cyclophosphamide, prednisone, and vincristine	Total
Dominant disease				
Visceral†	8/16	5/9	5/10	18/35
Osseous	0/1	2/5	0/1	2/7
Soft tissue‡	2/3	1/2	0/1	3/6
Postmenstrual category (yrs)				
<1	3/4	1/1	1/2	5/7
1–5	2/4	2/3	0/3	4/10
5–10	0/2	3/4	2/3	5/9
>10	5/10	2/8	2/4	9/22
Free interval (yrs)§				
<1	4/9	4/7	3/6	11/22
1–5	5/9	3/7	2/6	10/22
>5	1/2	1/2	0/0	2/4
Total	10/20	8/16	5/12	23/48

* No. of responses/total No. of patients.
† Includes lung, liver, abdominal masses, CNS.
‡ Indicates local (skin) recurrence and lymph node involvement.
§ From time of mastectomy to recurrent disease.

clinically tolerable toxic effects. Interim examinations included leukocyte counts twice weekly and platelet counts weekly. Re-evaluation occurred 4 weeks after the start of treatment for the group receiving the multiple-drug program and 3–4 weeks after the start of treatment for the group receiving adriamycin.

At the time of re-evaluation, the pretreatment studies were again performed. If objective regression had occurred, the patients were re-treated with the same program. If the first treatment had failed, the patient was crossed over to the other treatment program. When the disease remained stable, objectively and subjectively, the initial program was continued until evidence of progressive metastatic disease was noted. Patients who failed to respond to both chemotherapeutic programs were removed from the study.

In the group of patients with visceral and soft tissue metastasis an objective regression was defined as a reduction in the size of the malignant lesion(s) by at least 50% of the product of the greatest perpendicular diameters. If osseous lesions were present, these must have remained stable or have diminished. In the event of hepatic metastasis, a reduction of one third in the sum of the linear measurements below each subcostal margin at the midclavicular line and at the xiphoid must have occurred. Progressive disease was defined as the appearance of any new lesion(s) or an increase of greater than 25% of any of the pre-existing lesions.

RESULTS

Table 1 indicates the responses observed with the various treatment programs employed in this study by discriminants, and table 2 indicates the overall response rate for each of the treatment programs.

Ten of 20 patients initially responded to the use of adriamycin; eight of the ten had visceral dominant disease. The median length of regression was 32 weeks (mean, 30.8 weeks). Four patients were crossed over to multiple-drug programs while continuing in remission and two of these patients with remissions are currently being maintained on the alternate treatment program employing multiple drugs. Thus far only one of nine patients has responded to adriamycin as secondary therapy after one of the multiple-drug programs failed as initial therapy.

Of 16 patients treated primarily with 5-FU, cyclophosphamide, and prednisone without vincristine, eight achieved objective regressions with a median duration of regression of 42 weeks (mean, 38.5 weeks). Five patients remain in regression at 52, 52, 47, 46, and 34 weeks, respectively.

Five of 12 patients responded to the multiple-drug program with the addition of vincristine as primary therapy with a median duration of regression of 40 weeks (mean, 38.8 weeks). Three patients remain in regression at 56, 56, and 40 weeks, respectively.

Patients receiving adriamycin were crossed over to a multiple-treatment program with the onset of progressive metastatic disease, evidence of congestive heart failure, or after having received a total dose of 550 mg/m^2 of adriamycin. Seven secondary responses occurred among 15 patients treated with the multiple-drug programs, but in four of these patients regressions were initially achieved utilizing adriamycin and were maintained with the multiple-drug programs. Of those 11 patients not exhibiting objective regressions at the time of crossover to multiple-drug exposure, only three responded.

The toxicity experienced with adriamycin was not considered excessive. As shown in table 3, 70% of the patients experienced leukopenia with an average nadir of leukocyte depression of 2200 cells/mm^3 with a mean onset of 13 days lasting a mean of 5.2 days. Only one patient had platelet depression and this was not clinically significant. Because of toxicity, however, seven of 20 patients had subsequent reduction of doses varying from 7% to 33%, usually because of excessive myelosuppression. In two patients adriamycin was stopped before the dose level reached 550 mg/m^2 because of questionable cardiotoxicity. Both of these patients had the suggestion of early or incipient cardiac failure. One patient died subsequent to this episode; however, the postmortem examination failed to support adriamycin as the causative factor. The other patient is being maintained on a

TABLE 2.—Response to adriamycin and to multiple drugs with or without vincristine*

Agents	Treatment	
	Primary	Secondary
Adriamycin	10/20	1/9
Multiple drugs		
Alone	8/16	4/7†
With vincristine	5/12	3/8†

*No. of responses/total No. of patients.

†Includes patients with adriamycin responses whose regressions were maintained by multiple-drug programs.

multiple-drug program with stability of disease at the present time. Alopecia occurred consistently after the administration of adriamycin and was usually complete.

The toxicity experienced in the combined treatment programs was as previously experienced and reported (1). As noted previously, more frequent myelosuppression and more pronounced alopecia were observed with the addition of vincristine to the program. Neurologic toxicity was limited to the group receiving vincristine. Of the 28 patients in the multiple-drug programs, nine had subsequent dose reductions of 5-FU and cyclophosphamide ranging from 5% to 25%, due again to excessive myelosuppression. Virtually all patients who received vincristine had dose reductions to a total dose of 1 mg on Days 1 and 5 of the treatment program after the initial course of treatment.

DISCUSSION

This study supports earlier evidence of the rather impressive activity of adriamycin in patients with advanced breast cancer. Adriamycin would appear to be at least as effective as any single agent previously reported and perhaps more active than many. The true rate of regression seen in this adriamycin experience was 50%; however, at 95% confidence limits, the regression rate could lie between 27% and 75%. The regimen we employed was reasonably well tolerated and with the exception of possible cardiotoxicity, this agent was no more clinically intolerable than most of the single agents or multiple-drug programs evaluated at our Institution.

The experience with the multiple-drug programs using 5-FU, cyclophosphamide, and prednisone with or without vincristine was essentially as previously reported and again raises some doubt as to the efficacy of adding vincristine to this treatment regimen in terms of an increased regression rate.

The adriamycin experience supports the need for the further investigation of this agent, perhaps in a lower dose range to avoid the possibility of cardiac toxicity. However, reports of O'Bryan et al (4) suggest that 45 mg/m² is too low a dose in good-risk patients. Investigations of adriamycin used in combination with other agents should also be exploited.

Secondary treatment with adriamycin in this study has thus far been disappointing; perhaps this has been so because of previous multiple-drug exposure in this group of pa-

TABLE 3.—Toxic effects of agents used in the two treatment programs*

Agents	Myelosuppression		Gastrointestinal effects †	Alopecia	Neuropathy	Cardiac toxicity
	Wbc (<3000 cells/mm³)	Platelets (<100,000 cells/mm³)				
Adriamycin	14/20	1/20	15/20	20/20	0/20	2/20‡
Multiple drugs						
Alone	11/16	5/16	14/16	12/16	0/16	0/16
With vincristine	10/12	1/12	10/12	12/12	12/12	0/12

*Primary treatment only. No. of patients experiencing toxic effects/total No. of patients.

†Includes stomatitis.

‡Not conclusively proven to be related to treatment with adriamycin.

tients rather than just a single agent. The number of patients, however, having received adriamycin as secondary treatment so far is small and a possibility still exists for the trial to be extended to achieve a 30% response rate. Further investigations should clarify the role of adriamycin as secondary therapy.

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Combination Chemotherapy of Bronchogenic Carcinoma With Cyclophosphamide (NSC-26271) and BCNU (NSC-409962) [1,2,3]

John E. Curtis [4,5,6]

SUMMARY

Cyclophosphamide (1000 mg/m²) and BCNU (100 mg/m²) were given intravenously every 21 days to patients with advanced lung cancer. Twenty (32%) of 62 evaluable patients had objective regression of disease including four patients who had complete remissions. Fourteen of 25 patients with small-cell and anaplastic carcinoma responded, as did one of five patients with large-cell carcinoma, one of 14 patients with squamous-cell carcinoma, two of 12 patients with adenocarcinoma, and two of six patients who were diagnosed by cytology alone. Response rate did not depend on the extent of disease or the dose of drugs administered. The major toxic effect was myelosuppression consisting mainly of neutropenia. Moderate cumulative myelosuppression was observed necessitating reduction in drug dose. Cyclophosphamide and BCNU therapy warrants further investigation in the management of small-cell and anaplastic carcinomas of the lung.

[Cancer Chemother Rep 58:883–888, 1974]

Treatment with cyclophosphamide results in objective disease regression and increased survival time in 20%–25% of patients who have bronchogenic carcinoma (1,2). Chemotherapy with BCNU is associated with a response rate of 11% in patients who have advanced primary lung cancer (1,3). The effectiveness of combination chemotherapy with cyclophosphamide and BCNU in patients with advanced bronchogenic carcinoma is described.

Improvement of the therapeutic activity of cyclophosphamide and BCNU in animal tumor systems has been obtained by using the drugs in combination (4). In the transplanted murine AK lymphoma and L1210 leukemia, the enhanced antitumor activity is synergistic (5, 6). Combination chemotherapy with cyclophosphamide and BCNU used against AK lymphoma resistant to single-drug treatment with cyclophosphamide or BCNU has suggested that the optimal molar ratio of cyclophosphamide to BCNU for therapeutic synergism is 10:1.[7] In addition, for these drugs to have a synergistic antitumor effect, they must be administered within 16 hours of each other (5).

METHODS

All patients eligible for this study had his-

[1] Received Apr 5, 1974; revised June 7, 1974; accepted June 21, 1974.

[2] Supported by The Ontario Cancer Treatment and Research Foundation.

[3] Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,-2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

BCNU: CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-. Supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, National Cancer Institute.

[4] Department of Medicine, Ontario Cancer Institute and University of Toronto, Canada.

[5] The author wishes to thank Drs. W. R. Bruce and D. E. Bergsagel for their encouragement and criticism, and Mrs. Joan Reid for her assistance in data analysis.

[6] *Reprint requests to:* Dr. J. E. Curtis, Ontario Cancer Institute, 500 Sherbourne St, Toronto, Canada, M4X 1K9.

[7] Bruce WR. Personal communication.

tologically or cytologically proven primary lung cancer (7). These patients had recurrent disease after radiation therapy or had disease which was not considered suitable for radiation therapy either because of too extensive intrathoracic disease or the presence of distant metastases. All patients had objective measurable disease. Only one patient had had prior chemotherapy and no patients had had radiation therapy within 1 month preceding the start of chemotherapy. Prior to chemotherapy, all patients had the following determinations taken: hemoglobin, white blood cell count and differential, platelet count, blood urea nitrogen, urinalysis, serum bilirubin, alkaline phosphatase, serum glutamic oxaloacetic transaminase, and serum protein electrophoresis. Roentgenograms and other laboratory tests were performed as necessary to document the extent of disease. The majority of patients had a bone marrow aspiration taken from a site that had not been subject to radiation treatment.

The dose of cyclophosphamide was 1000 mg/m^2 of body surface area and was injected into the tubing of an intravenous (iv) infusion of 5% dextrose and water. The dose of BCNU was 100 mg/m^2 and was given at the same time by iv push. Initial doses of 750 mg/m^2 of cyclophosphamide and 75 mg/m^2 of BCNU were given to two patients treated at the onset of the study, four patients whose age was greater than 70 years, and five patients who had hepatic function impairment. Peripheral blood counts were obtained on a weekly basis and repeat courses of treatment were given at 21-day intervals until there was evidence of disease progression. If the nadir of the granulocyte count was less than 500 cells/mm^3, the subsequent doses of both drugs were reduced by 25%. If at 21 days the leukocyte count was less than 3000 cells/mm^3, or the platelet count was less than 100,000 platelets/mm^3, therapy was delayed for 1 week or until the counts had risen above these levels and the dose was then reduced by 25%.

Patients were evaluated clinically every 3 weeks. A chest roentgenogram was obtained if necessary every 3 weeks. Other investigations were performed at appropriate times to evaluate response to treatment.

A response was defined as a more than 50% reduction in the sum of the tumor areas determined by multiplying the two greatest diameters of all measurable lesions. A reduction in liver size was considered a response if the distance between the lower margin of the liver and the costal margin at the lateral border of the right rectus muscle decreased by 30% or more. In the case of roentgenographically defined lesions that could not be measured, only total or major reductions in the size of the mass were considered responses. The above responses occurred in the absence of an increase in the size of any mass or the appearance of new lesions and persisted for a minimum of 6 weeks. Patients were considered to have progressive disease when new lesions appeared or when there was an increase in tumor size. Chemotherapy was then discontinued.

RESULTS

Sixty-four patients with primary lung cancers were treated with cyclophosphamide and BCNU. Two patients were not evaluable because of inadequate followup. Responses were observed in 20 of the remaining 62 patients (table 1). Chemotherapy induced responses in 14 of 25 patients with small-cell and anaplastic tumors of the lung, but induced responses in only six of 37 patients with other types of primary lung cancer ($P < 0.01$). One patient with large-cell carcinoma, one with adenocarcinoma, and two with small-cell carcinomas had complete remissions. Response to chemotherapy with BCNU and cyclophosphamide occurred shortly after starting chemotherapy, if at all. At the time of the first followup visit 21 days after the initial dose of chemotherapy, improvement was detected in all of the patients who showed a response except for two (table 1).

Objective regression of disease occurred in five of 12 patients who were treated initially with reduced doses of cyclophosphamide and BCNU, and in 15 of 50 patients who were treated initially with full doses of cyclophosphamide and BCNU.

Four months after treatment was started, the survival rates of the two groups of pa-

TABLE 1.—Summary of objective responses to chemotherapy

Histologic type of carcinoma	Total No. of patients	Patients responding to chemotherapy (No./%)	No. of patients with responses after treatment for—		
			3 wks	6 wks	9 wks
Small-("oat") cell and anaplastic	25	14/56	14	0	0
Large-cell	5	1/20	1	0	0
Squamous-cell	14	1/7	1	0	0
Adenocarcinoma	12	2/17	2	0	0
Diagnosed by cytology only	6	2/33	0	2	0
All cell types	62	20/32	18	2	0

tients responding to chemotherapy were significantly better than those of the nonresponders ($P<0.05$) (fig 1). However, by 5 months from the onset of treatment there was no significant difference in the survival rates of the two groups of patients.

Regression of disease occurred in ten of 26 patients who had disease that was confined to the thorax and neck nodes (table 2) and was also observed in ten of 36 patients who had disease that was documented outside the thorax. This difference in response rate was not statistically significant. Seven of 22 patients with disease that was recurrent in an irradiated area responded to chemotherapy (table 3). No clinical improvement was observed in the neurologic symptoms and signs of nine patients who had metastases to the brain. One of ten patients had regression of bony disease as indicated by decreased pain and radiographic evidence of healing of the bone. Disease at other anatomic sites responded to chemotherapy with about equal frequency.

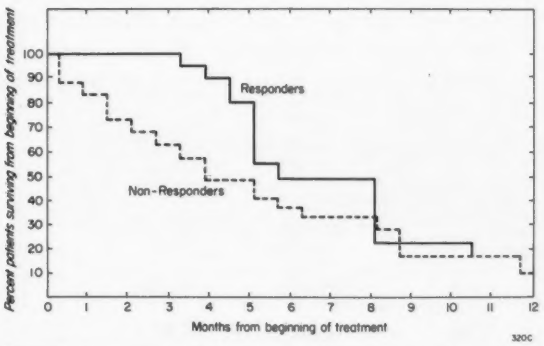

FIGURE 1.—Comparison of duration of survival from start of treatment for patients responding and for patients not responding to chemotherapy.

Bone marrow aspirations were done in 52 of the patients and 14 of these aspirates contained tumor cells. Eleven of 23 aspirates obtained from patients with small-cell carcinoma were positive for malignant cells compared with none of four from patients with large-cell tumors, none of 12 from patients with squamous-cell carcinoma, two of 11 from patients with adenocarcinoma, and one of two from patients diagnosed by cytology alone.

The patients who responded to chemotherapy had a shorter duration of disease before starting chemotherapy than did the patients in the nonresponding group (table 4). Only one of the patients in the responding group had a previous surgical resection. These observations reflect the predominance of patients with small-cell and anaplastic tumors in the group who responded to chemotherapy. Chemotherapy was tolerated similarly by both groups of patients as indicated by the similar degrees of myelosuppression after chemotherapy (table 5).

All patients had some nausea and vomiting. This usually occurred between 2 and 12 hours after chemotherapy and rarely persisted for more than 24 hours. Sixty-two percent of the courses of chemotherapy were given in the outpatient clinic. Forty-one patients had alopecia, four patients had phlebitis, and two patients had hemorrhagic cystitis. Bronchopneu-

TABLE 2.—Relationship of chemotherapeutic response to extent of disease

Disease site	Total No. of patients	Patients responding to chemotherapy (No./%)
Confined to thorax	26	10/38
Extrathoracic	36	10/28

TABLE 3.—Location of clinically detectable disease and response to chemotherapy at these sites

Site of clinically detectable disease	Total No. of patients	Patients responding to chemotherapy (No./%)
Brain	9	0/0
Local lung recurrence	22	7/32
Other lung involvement	12	5/42
Mediastinum (including heart)	41	15/37
Pleura	12	2/17
Neck nodes	20	12/60
Liver	21	9/43
Bone	10	1/10
Skin	7	2/29
Other*	9	2/22

*Three patients with axillary masses; four with abdominal masses; one with breast mass; and one with esophageal invasion.

monia occurred in two patients during the study and one patient died of pneumonia while severely neutropenic. Depression of the hematopoietic system consisted mainly of a decrease in the absolute granulocyte count (table 5 and figs 2–4). The changes followed a cyclic pattern associated with the administration of intermittent high doses of cyclophosphamide, but tended to become lower with successive courses of treatment (8,9). This probably reflected the delay in myelosuppressive effects of BCNU and necessitated reduction in the doses of drugs used in the third or subsequent courses of chemotherapy or both. No patient had granulocytopenia of less than 1000 cells/mm^3 lasting more than 7 days.

TABLE 4.—Characteristics of patients treated with cyclophosphamide and BCNU

	Responders	Nonresponders
No. of patients	20	42
Age (yrs): median/range	56/37–72	61/43–73
No. of women	1	4
Duration of disease from diagnosis to onset of chemotherapy (days): median/range	32/0–1029	98/7–205
Patients having prior:		
Surgery	1	10
Radiation	8	14
Both	0	7

DISCUSSION

In this study patients with advanced lung cancer were treated with full therapeutic doses of cyclophosphamide and BCNU. The recommended dose of BCNU ranged from 200 to 250 mg/m^2 given every 6 weeks (10). By dividing the dose of BCNU it is possible to combine it with cyclophosphamide in a way that animal studies indicated was optimal for obtaining synergism (5,6). This schedule also enabled the administration of cyclophosphamide in large intermittent doses that have been considered therapeutically most effective (11).

The clinical importance of the histologic grading of primary lung cancer is evident from

TABLE 5.—Myelosuppressive effects of chemotherapy with cyclophosphamide and BCNU

	No. of patients with—*			
	Granulocyte nadir of—(cells/mm^3)		Platelet nadir of—(platelets/mm^3)	
	<1000	<500	<150,000	<100,000
Responders	11/20	7/20	7/20	3/20
Nonresponders	17/31	9/31	6/32	4/32

* No. of patients affected/No. of patients evaluated.

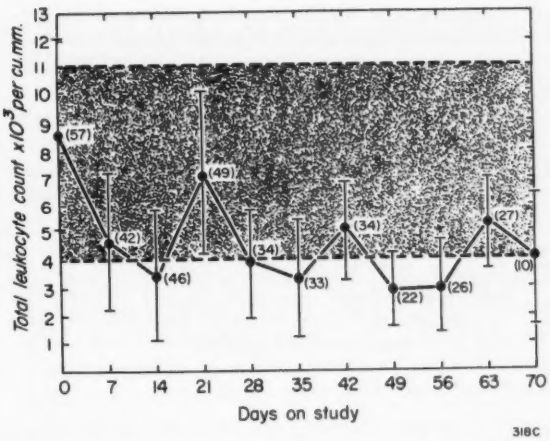

FIGURE 2.—Effect of chemotherapy on total leukocyte count. Shaded area = normal range; points and brackets = mean and standard deviation respectively; Nos. in parentheses = No. of studies performed.

the results of this study (12). The patients with small-cell (oat-cell) and anaplastic tumors responded frequently to combination chemotherapy with cyclophosphamide and BCNU. Responses of patients with other tumor cell types were no more frequent than expected from treatment with cyclophosphamide alone (1). A controlled clinical trial of combination chemotherapy with cyclophosphamide and BCNU is necessary to determine if the anti-

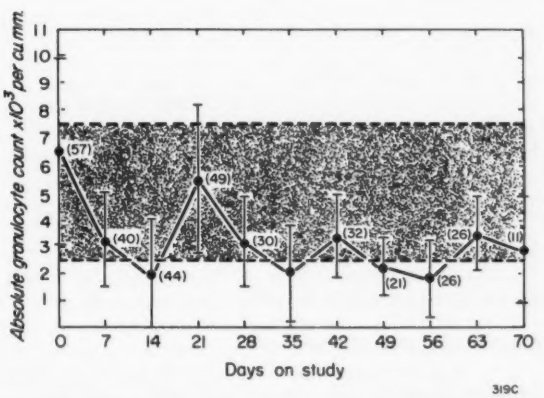

FIGURE 3.—Effect of chemotherapy on absolute granulocyte count. Symbols same as in figure 2.

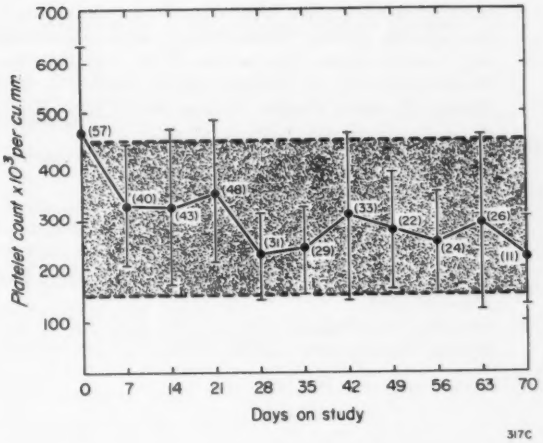

FIGURE 4.—Effect of chemotherapy on platelet count. Symbols same as in figure 2.

tumor activity of this drug combination in small-cell carcinoma of the lung is more than additive. Recent reports of intensive combination chemotherapy and radiation therapy have indicated that the survival rates of patients with lung cancer can be improved (13). Chemotherapy with cyclophosphamide and BCNU for patients with small-cell or anaplastic tumors of the lung in combination with radiotherapy warrants investigation.

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Clinical Trial of Isophosphamide (NSC-109724)—Results and Side Effects[1,2]

D. N. Bremner, J. StC. McCormick, and J. W. W. Thomson[3,4,5]

SUMMARY

Sixteen patients with advanced malignancies were treated with isophosphamide, an analog of cyclophosphamide. Six patients evidenced remission of their disease and all patients had significant drug-related side effects including glycosuria which has not been previously reported. Most patients required urethral catheterization because of severe hemorrhagic cystitis. Two patients died during the study. It was concluded that isophosphamide was effective, though toxic, in the treatment of advanced cancer.

[Cancer Chemother Rep 58:889–893, 1974]

Isophosphamide is a new antineoplastic compound chemically similar to cyclophosphamide. Isophosphamide is an oxazaphosphorine analog of cyclophosphamide that differs from the parent substance in that one of the chloroethyl groups is attached to the endocyclic nitrogen atom, whereas in cyclophosphamide both chloroethyl groups are attached to the exocyclic nitrogen atom (fig 1). This substance, when activated enzymatically in the liver, will exert a cytotoxic or an alkylating effect (1) and has been shown to be more effective than cyclophosphamide in the treatment of some animal tumors (2).[6]

The present trial was undertaken to study the efficacy of the substance when used in the treatment of patients with advanced cancer after conventional methods of treatment had been discontinued due to cessation of effectiveness.

MATERIAL AND METHODS

Sixteen patients, five men and 11 women, with advanced malignancies received the drug. Table 1 provides information on the patients, the sites of primary and secondary tumors, and the previous treatment. All patients had biopsy-proven neoplasms.

Before treatment, every patient had a complete blood cell count, urea and electrolyte determinations, liver function tests, plasma protein, serum amylase, and uric acid estimations, as well as urinalysis and a glucose tolerance test. These estimations were repeated frequently during and after each course of treatment.

Isophosphamide, at a daily dose of 1.2–3.6 g/m^2 of body surface area, was given by intravenous (iv) infusion on 2–3 consecutive days. This course of medication was repeated up to three times at 4–6-week intervals. In most patients, because of the severe hemorrhagic cystitis caused by the hydrolysis prod-

[1] Received Apr 22, 1974; revised July 9, 1974; accepted July 19, 1974.

[2] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

[3] Chalmers Hospital, Edinburgh, Scotland.

[4] The authors wish to accord their grateful thanks to Dr. J. M. Simister of W. B. Pharmaceuticals Ltd., for giving them the opportunity of using the drug and for his encouragement during the trial.

[5] *Reprint requests to:* J. W. W. Thomson, Chalmers Hospital, Edinburgh EH3 9HQ, Scotland.

[6] Friedman MA, and Carter SK. Clinical brochure: ifosfamide (NSC-109724), Sept 1970. Prepared by the DCT, NCI, Bethesda, Md.

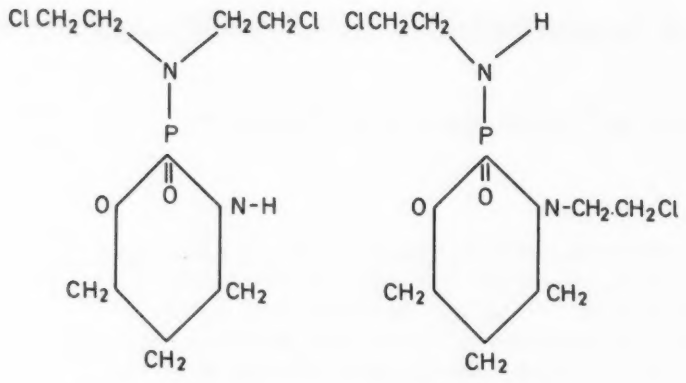

FIGURE 1.—Chemical structures of cyclophosphamide and isophosphamide.

ucts of the compound, urethral catheters were inserted, a high fluid intake was ensured, and urine samples were alkalinized by an iv administration of 500 ml of 1.26% sodium bicarbonate before and after each dose. A potassium citrate mixture was also given orally and the patients were sedated with chlorpromazine or diazepam. When necessary, antiemetics, antispasmodics, and analgesics were given. The total dose for each patient varied from 3.6 to 19 g/m² of body surface area.

All patients were followed up at monthly intervals at a special chemotherapy outpatient clinic, and were examined by two observers.

Response to treatment was graded as follows:

Complete response.—Complete disappearance of all clinical disease for at least 3 months.

Partial response.—A greater than 50% objective decrease in the size of all measurable lesions for at least 3 months.

No change.—A less than 50% objective decrease in the size of all measurable lesions or no change.

Progression.—The disease advanced unchanged.

RESULTS

Complications and Side Effects of the Drug

Deaths.—Two patients died during the study. One patient, with reticulum cell sarcoma, became comatose after two doses of 3.6 g/m² of body surface area and died 12 days later. In this patient the hemoglobin value and the white blood cell (wbc) and platelet counts fell sharply; the blood urea nitrogen (BUN) level rose to 350 mg/100 ml, and the uric acid level rose to 25 mg/100 ml. A postmortem examination confirmed the diagnosis and revealed marked pallor of the renal cortices which were wider than normal and edematous. There was no evidence of hemorrhage in the kidneys, ureters, or bladder. In the other patient, who had squamous carcinoma of the kidney, a 103° F pyrexia developed after two doses of 3.6 g/m² and he died of septicemia 7 days later. The wbc count had fallen from 7700 to 300 cells/mm³, the BUN level had risen to 340 mg/100 ml, and the uric acid level had risen to 19 mg/100 ml. Postmortem examination revealed that one kidney was completely replaced by tumor and the other kidney, containing granular and protein casts, showed extensive areas of autolysis with widespread tubular damage.

Side effects.—All 16 patients had moderate to severe dysuria and frequency, and five pa-

TABLE 1.—Summary of information on clinical trial of isophosphamide

Patient No.	Sex/age	Primary site	Secondary sites	Previous treatment	Total dose (g/m^2 of body surface area)	Time in mos from—		Responses			
						Diagnosis to start of treatment	Start of treatment to conclusion	Complete	Partial	No change	Progression
1	F/63	Ovary	Peritoneum	5-Fluorouracil	3.6	4	3 (died)	–	–	–	+
2	F/51	Ovary	Peritoneum	Radiotherapy, thio-TEPA	7.2	9	5 (died)	–	+	–	–
3	F/40	Ovary	Peritoneum, pleura	ThioTEPA	10.2	15	4 (died)	–	+	–	–
4	F/49	Pancreas	Local	Biliary bypass	14.3	3	7 (died)	–	–	–	+
5	M/46	Pancreas	Local	Biliary bypass	10.8	1	4 (died)	–	–	–	+
6	F/69	Pancreas	Local	Biliary bypass	19.1	1	27 (alive)	+	–	–	–
7	F/60	Hypernephroma	Chest	Cyclophosphamide	14.3	8	5 (died)	–	–	–	+
8	M/49	Hypernephroma	Bone	None	14.3	24	6 (died)	–	–	–	+
9	M/55	Kidney (squamous cell carcinoma)	Local	None	7.2	2	0 (died during the study)	–	–	–	+
10	M/42	Colon	Peritoneum, liver	Anterior resection	14.3	7	6 (died)	–	–	–	+
11	F/50	Colon	Peritoneum	Cyclophosphamide, *N*-deacetylthiocolchicine	11.9	3	4 (died)	–	+	–	–
12	F/71	Rectum	Bone, lung, liver	Abdominoperineal resection	7.2	12	1 (died)	–	–	–	+
13	F/47	Breast	Peritoneum, chest, liver, nodes	Radiotherapy, thio-TEPA, cyclophosphamide, nandrolone decanoate	14.3	30	23 (died)	–	+	–	–
14	F/75	Buttock (squamous cell carcinoma)	Nodes	Radiotherapy	8.0	7	18 (alive)	–	+	–	–
15	F/46	Melanoma	Eye, skin	Hyperthermia	17.9	84	3 (died)	–	–	+	–
16	M/46	Reticulum cell sarcoma	Bone, chest	None	7.7	1	0 (died during the study)	–	–	–	+

tients required antibiotics for bacteriologically proven urinary tract infection. Ten patients had hematuria and one patient complained of urinary incontinence. Eight patients had nausea and vomiting, 12 felt unwell, and two were confused. Twelve patients had moderate to severe alopecia; six of the nine affected women required wigs. One patient had a stress ulcer and a brisk hematemesis, but her platelet count had remained at 225,000 platelets/mm^3 during the study.

Hematologic and biochemical changes.—Hemoglobin levels fell by more than 2 g/100 ml in five patients, the wbc count fell to less than 3000 cells/mm^3 in ten patients (< 1000 cells/mm^3 in four of the ten patients), and the platelet count fell to less than 20,000 platelets/mm^3 in three patients. The BUN rose to between 70 and 120 mg/100 ml in five patients and rose to around 300 mg/100 ml in the two patients who died during the study. In one patient, a serum sodium level of 155 mEq/liter was recorded but otherwise the serum sodium, potassium, chlorides, and bicarbonate levels were within normal limits in all patients. Serum amylase, bilirubin, serum glutamic oxaloacetic transaminase, and serum glutamic pyruvic transaminase levels were normal in all patients except those with carcinomas of the pancreas. Alkaline phosphatase (AP) levels were abnormally high in six patients, two of whom had carcinoma of the pancreas. Total protein values fell to low levels in three patients and albumin levels fell in eight patients. Serum uric acid levels were elevated in nine patients. These changes in AP, protein, and uric acid levels were not considered to be related to changes in disease status. Glycosuria was noted in ten patients, seven of whom had normal glucose tolerance tests before and after treatment. Two patients did not have the test performed and one patient had a peak of 170 mg/100 ml that was not sustained. Casts were noted in the urine of only one patient. Significant proteinuria was encountered in six patients.

Response and Survival

One patient had a complete response and five patients had partial responses to treatment with isophosphamide. There was no change in one patient, and disease progressed in nine patients.

Two patients died during the study, three died within 3 months, eight died between 3 and 9 months, one died at 23 months, and two patients are still alive 18 and 27 months, respectively, after the start of treatment with isophosphamide, although both of these patients now have evidence of progressive malignancy.

DISCUSSION

The rate of response recorded in this group of patients is similar to that recorded in other series (2,4), ie, around 40%, but the present rate is higher than that encountered by Wang et al (6), who reported four partial responses in 21 patients. More than half of the patients, however, had childhood tumors and this may explain the discrepancy between the response rates.

These responses compare favorably with those reported for cyclophosphamide. The difference may, of course, be due to the different method of administration, ie, bolus injection as compared to daily oral doses. We were unable to determine any clear relationship between dose and response rate, and the number of patients with different tumors was too small to allow interpretation of the response of each tumor type to the drug.

The side effects encountered were similar to those previously reported (4) with the exception of glycosuria which has not been recorded elsewhere. Glycosuria occurred in 60% of the patients but was associated with normal serum amylase and normal glucose tolerance tests and, therefore, presumably reflects a state of lowered renal threshold to sugar (another manifestation of the nephrotoxicity of the drug). Other manifestations of nephrotoxicity were hematuria, uremia, and elevated serum uric acid levels. The presence of casts in the urine and the occurrence of hypocalcemia were not notable features as reported elsewhere (4,6). Marrow toxicity was exhibited by decreases in hemoglobin levels, decreases in wbc and platelet counts, and elevated uric acid levels. The other possible causes for the elevated serum uric acid levels

are tumor destruction with release of nucleoproteins, due to the chemotherapeutic agent, and progressive renal failure. The hair loss, hemorrhagic cystitis, and upper gastrointestinal symptoms were mainly moderate to severe and were recorded in most cases. The two deaths possibly attributable to therapy occurred because of kidney damage and marrow depression/kidney damage. No clear relationship between the dose of isophosphamide and the severity of the side effects could be established.

In conclusion, it was felt by the authors that isophosphamide was as effective as other alkylating agents in the treatment of advanced malignancy. However, it proved toxic and complex in use and it may be that better results with fewer side effects will be obtained if, as suggested (5), the alkylating effect of the drug is potentiated with phenobarbital.

LIST OF COMPOUNDS

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8

Isophosphamide: NSC-109724; CAS reg. No. 3778-73-2; 1,3,2-oxazaphosphorine, 3-(2-chloroethyl)-2-[(2-chloroethyl)amino] tetrahydro-, 2-oxide; Z 4942

Nandrolone decanoate: CAS reg. No. 360-70-3; Decadurabolin

N-deacetylthiocolchicine: NSC-9170; CAS reg. No. 2731-16-0; Thio-colciran; *N*-desacetyl thiocolchicine

ThioTEPA: NSC-6396; CAS reg. No. 52-24-4; phosphine sulfide, tris(1-aziridinyl)-

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Phase I Study of Twice Weekly 5-Azacytidine (NSC-102816) [1,2,3]

William Ralph Vogler, Sureyya Arkun, and Enrique Velez-Garcia [4,5]

Writing Committee for the Southeastern Cancer Study Group

SUMMARY

A phase I study of 5-azacytidine given by rapid intravenous injection twice weekly was conducted in 25 patients with metastatic cancer and normal bone marrows. Nineteen patients were in the study at least 6 weeks. Doses were started at 50 mg/m² and were increased by 50-mg/m² increments in subsequent sets of patients. Nausea and vomiting occurred in all patients at doses of 100 mg/m² or more. The major hematologic toxicity was granulocytopenia occurring in ten of 11 patients treated with doses of 150 mg/m² or more.

[Cancer Chemother Rep 58:895–899, 1974]

5-Azacytidine, an analog of cytidine, was synthesized by Piskala and Sorm in 1964 (1). The drug has antitumor activity in a variety of animal tumors (2–4). In vivo studies indicate that the drug is S-phase specific (5) and is incorporated into RNA and DNA (4). Responses have been observed in clinical studies using single daily rapid intravenous (iv) injections for periods of 5–10 days in patients with metastatic cancer and acute leukemia (6–9). The major limiting toxic effects have been severe nausea and vomiting (7). Hematologic toxicity was primarily neutropenia (6). Studies in L1210 leukemia [6] indicated that the most effective schedule was continuous treatment given daily on Days 1–5 (240% increase in lifespan) or Days 1–9 (250% increase in lifespan). When given every fourth day there was a 207% increase. Therefore, the Southeastern Cancer Study Group initiated a phase I study of twice-weekly 5-azacytidine to complement work already under way and to reduce perhaps the frequency of nausea and vomiting.

MATERIALS AND METHODS

Patient selection.—Patients with advanced metastatic cancers unresponsive to surgery or radiation were eligible for the study. Their life expectancy had to be at least 6 weeks. Normal bone marrow, blood cell counts, renal, and hepatic function were required. Patients

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[2] Supported by Public Health Service research grants CA-03227 and CA-11263 (Emory University School of Medicine, Atlanta, Ga), CA-14299 (Veterans' Administration Hospital, Brooklyn, NY), and CA-12223 (University of Puerto Rico School of Medicine, San Juan), from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] 5-Azacytidine: *s*-triazin-2(1*H*)-one, 4-amino-1-β-D-ribofuranosyl-; CAS reg. No. 320-67-2. Supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, NCI.

[4] The following members of the Southeastern Cancer Study Group participated in this study: *William Ralph Vogler, James Keller, Charles M. Huguley, Lawrence E. Cooper,* and *Yick-Kwong Chan,* Emory University School of Medicine, Atlanta, Ga; *Sureyya Arkun,* Veterans' Administration Hospital, Brooklyn, NY; and *Enrique Velez-Garcia,* University of Puerto Rico School of Medicine, San Juan.

[5] *Reprint requests to:* William R. Vogler, MD, Department of Medicine, Emory University School of Medicine, Woodruff Memorial Bldg, Atlanta, Ga 30322.

[6] Broder LE, and Carter SK. Clinical brochure: 5-azacytidine (NSC-102816). Prepared by the NCI, Bethesda, Md, Oct 1970.

had to have recovered from any toxicity from prior radiation therapy or chemotherapy.

Pretreatment studies.—Pretreatment studies included history, physical examination, and the recording of any measurable neoplastic lesions. The following tests were required: bone marrow aspiration and biopsy, hemogram, serum glutamic oxaloacetic transaminase (SGOT) or serum glutamic pyruvic transaminase, alkaline phosphatase, serum proteins, prothrombin time, blood urea nitrogen (BUN), and creatinine or creatinine clearance.

Studies during therapy.—Blood cell counts were repeated twice weekly. Hepatic and renal function tests and prothrombin time were repeated at weekly intervals.

Treatment regimens.—In all instances the drug was administered by a rapid iv injection. The plan of treatment was as follows: The first three patients were to be treated with 50 mg/m^2 twice weekly. If no toxicity occurred after 8 weeks of therapy the next set of three patients were to be treated with 100 mg/m^2 twice weekly, and doses in subsequent sets of three patients were to be escalated by 50-mg/m^2 increments until toxicity occurred. It was planned to treat all patients for a period of 8 weeks at a constant dose with 2 additional weeks of followup. Once toxicity was observed it was planned to treat six patients at that dose level before escalation.

Evaluation of response.—Patients with measurable evidence of tumor were evaluated according to the reduction in tumor size. An excellent response was indicated by a disappearance of all evidence of disease. A good response was indicated by a greater than 50% reduction and a fair response was indicated by a greater than 20% reduction in the size of measurable tumors. A questionable improvement was indicated by a less than 20% reduction or subjective improvement.

Evaluation of toxicity.—Hematologic toxicity was graded as follows:

Hemoglobin: 1 = a fall of 3–4.9 g/100 ml; 2 = a fall of 5 or more g/100 ml.

Granulocytes: 1 = a fall below 1500 cells/mm^3; 2 = a fall below 750 cells/mm^3.

Platelets: 1 = a fall below 100,000 cells/mm^3; 2 = a fall below 50,000 cells/mm^3.

Grade 3 granulocyte and platelet toxicity was reserved for life-threatening situations.

Hepatic toxicity: 1 = a rise in SGOT or alkaline phosphatase by 50%; 2 = a rise in SGOT or alkaline phosphatase by 100%.

Renal toxicity: a rise in BUN or creatinine or a fall in creatinine clearance below normal. This toxicity was not graded.

Gastrointestinal toxicity: the presence of nausea, vomiting, and diarrhea was recorded and graded as either mild, moderate, or severe.

RESULTS

The institutions participating in this study were Emory University, Brooklyn Veterans' Administration Hospital, and the University of Puerto Rico. Of 25 patients entered in the study, 19 completed at least 6 weeks of therapy. Table 1 shows the gastrointestinal and hematologic toxic effects as related to dose. Six patients were entered in the study at a dose of 50 mg/m^2 and each completed at least 8 weeks of therapy. Three of these patients experienced mild nausea and vomiting. There was no hematologic toxicity. All patients received 100% of the planned dose. Five patients were treated at a dose of 100 mg/m^2 and four were in the study for at least 6 weeks. All five had moderate nausea and vomiting. One patient had a greater than 3-g/100 ml fall in hemoglobin. One patient who died of infection had a granulocyte count of less than 750 cells/mm^3 and another had a count of less than 1500 cells/mm^3. These five patients received 90% of the projected dose. Seven patients were treated at a dose of 150 mg/m^2 and all completed at least 6 weeks of treatment. All patients had moderate to severe nausea and vomiting with each dose of treatment. Three patients had a fall in hemoglobin which was thought to be secondary to the drug and all seven had granulocyte toxicity (three at grade 2 and four at grade 1). One patient with grade 1 toxicity died of infection. These patients received 75% of the intended drug dose. At the 200-mg/m^2 level there were seven patients entered and four who were in the study for at least 6 weeks. All seven experienced moderate to severe nausea and vomiting. One patient

TABLE 1.—Relationship of 5-azacytidine dose to toxicity

Dose (mg/m²)	No. of patients— Entered	No. of patients— On study for 6 wks	No. of patients with— Nausea and vomiting	Fall in hemoglobin level (>3g/100 ml)	Platelet count <140,000 cells/mm³	Granulocyte count (cells/mm³) <1500	Granulocyte count (cells/mm³) <750	Projected dose (%)
50	6	4	3	0	0	0	0	100
100	5	4	5	1	0	1	1	90
150	7	7	7	3	0	4	3	75
200	7	4	7	1	0	0	3	67

had grade 1 hemoglobin toxicity and three experienced granulocytopenia of less than 750 cells/mm³. These patients received 67% of the total intended dose.

There were no hepatic or renal toxic effects attributable to the drug. Prothrombin times were unchanged. The most troublesome side effects were nausea and vomiting which were moderate to severe, occurred approximately 90 minutes after administration of the drug, and persisted for several hours. They were sometimes accompanied by diarrhea.

Granulocytopenia was the only significant hematologic toxic effect and was dose related as illustrated in figure 1. The onset of granulocytopenia was gradual, occurring between 4 and 6 weeks after the start of treatment. In most instances treatment was stopped when the granulocyte count fell below 2000 cells/mm³. The counts continued to fall, and 1–3 weeks after stopping treatment the levels were below 1500 cells/mm³ in nine of the 11 patients receiving 150 mg/m² or more. In those cases treatment was stopped between 4 and 7 weeks, the nadir occurred between 6 and 7 weeks (range, 5–8.5 weeks), and recovery occurred in 1 or 2 weeks (range, 1–3 weeks).

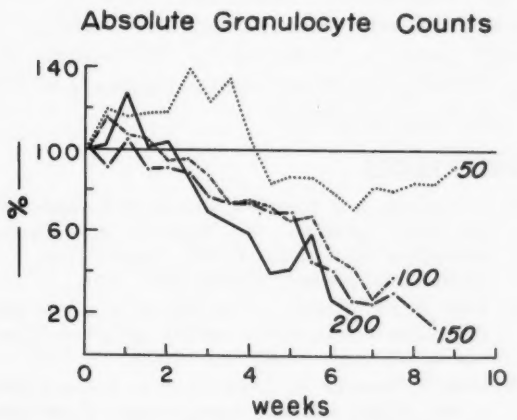

FIGURE 1.—Means of the granulocyte counts expressed as percentages of the pretreatment values for each dose schedule of 5-azacytidine. 50–200 indicate doses in milligrams per square meter.

Table 2 shows the types of tumors treated and the patient responses. There were seven patients with carcinoma of the colon. All received at least 4 weeks of treatment without a response. There were four patients with metastatic carcinoma (primary unknown) who were in the study at least 4 weeks with no response. One patient with carcinoma of the lung treated with 100 mg/m² had neither response nor hematologic toxicity. Treatment was withheld for 3 weeks and resumed at 150 mg/m²; a questionable response was observed. One asbestos worker with mesothelioma involving the abdomen and the pleura with signs of partial intestinal obstruction was treated for 7 weeks at 150 mg/m²; he responded with a disappearance of abdominal pain and signs of obstruction. Granulocytopenia developed and treatment was withheld. Symptoms recurred after several weeks. He was then treated with 200 mg/m². Treatment was stopped after 5.5 weeks because of granulocytopenia. Again he showed a response to therapy. He was given a third course of treatment with a favorable response. The total duration of his response was about 9 months. One patient with pulmonary involvement from metastatic breast cancer showed improvement in respiratory symptoms but no objective evidence of tumor regression. Thus, only three of 21 patients treated for at least 4 weeks had some suggestion of an antineoplastic effect.

TABLE 2.—Response to treatment according to primary site

Site	No. of patients— Entered	Treated > 4 wks	Responding*
Gastrointestinal tract (adenocarcinoma)			
Colon	7	7	0
Pancreas	1	1	0
Stomach	1	0	0
Esophagus	1	0	0
Hepatoma	1	0	0
Adenocarcinoma, primary unknown	4	4	0
Lung			
Giant cell	1	1	0
Squamous cell	2†	2	1
Melanoma	1	0	0
Breast	1	1	1
Endometrium	1	1	0
Liposarcoma	1	1	0
Mesothelioma	3†	3	2

*All responses were categorized as questionable.

†One patient treated at 2 different dose schedules.

Nine patients died while entered in the study: progressive disease, three patients; pulmonary embolus, three patients; infection, two patients; and gastrointestinal bleeding, one patient. The latter death was not thought to be drug related.

DISCUSSION

This phase I study has established the fact that 5-azacytidine given in doses of 150 mg/m^2 for approximately 6 weeks to patients with normal bone marrows will produce granulocytopenia as the major hematologic toxicity. The decline in granulocytes is gradual, reaching a nadir 1–2 weeks after the drug is stopped. There was minimal effect on hemoglobin concentration and none on the platelet count. We recommend that the maximum dose of 5-azacytidine given by this schedule should not exceed 150 mg/m^2. Treatment should be stopped when the absolute granulocyte count drops to less than 2000 cells/mm^3 because a further reduction was almost always observed over the next 1–3 weeks. This selective effect on granulocytes would suggest that 5-azacytidine might be an effective agent in acute granulocytic leukemia, producing less thrombocytopenia than other agents. Therapeutic benefit has been observed in acute leukemia (8) and further exploration is indicated.

Unfortunately, the limiting factor in administering 5-azacytidine by rapid iv injection is the severe degree of nausea and vomiting occasionally associated with diarrhea which follows each injection. These effects are only partially controlled by antiemetics. Despite these side effects most patients are willing to continue treatment.

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Phase I Clinical Trial of Weekly Administration of 4′-Demethylepipodophyllotoxin 9-(4,6-*O*-Ethylidene-β-D-glucopyranoside) (NSC-141540; VP-16-213) [1,2]

Patrick J. Creaven, Stephen J. Newman, Oleg S. Selawry, Martin H. Cohen, and Aron Primack[3,4,5]

SUMMARY

An initial clinical phase I trial of 4′-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside) (NSC-141540) has been carried out in 40 patients at dose levels of 20–290 mg/m² of body surface area using weekly intravenous administration and a modified Fibonacci search. Sporadic non-dose-related toxicity consisting of leukopenia (four of 17 patients), nausea and vomiting (dose limiting in two of 17 patients), thrombocytopenia (one of 17 patients), and cardiac arrhythmia (one of 17 patients) was seen at doses between 20 and 130 mg/m². Leukopenia was seen in 23 of 23 patients treated at doses of 170, 220, and 290 mg/m². Thrombocytopenia was seen in three of 23 patients at these dose levels; there were no other dose-limiting toxic effects. The drug is safe to use because of the predictable hematologic toxicity and the shallow dose-response curve. A starting dose of 290 mg/m²/week is recommended for phase II trials in patients who have not had extensive prior chemotherapy and/or radiotherapy, but downward dose modification will be necessary in most patients treated at this dose.

[Cancer Chemother Rep 58:901–907, 1974]

NSC-141540 is one of a series of semisynthetic podophyllotoxin derivatives developed by Sandoz Ltd., Basel, Switzerland, which have been found to be active against experimental tumor systems (1) and which have undergone clinical trial in man (2). NSC-141540 has been investigated in phase I clinical trials using two schedules: daily × 7 and twice weekly for 3 weeks (3). On the twice weekly for 3 weeks schedule, the maximum tolerated dose was 86 mg/m² (weekly dose, 172 mg/m²). We are reporting the results of an initial clinical phase I trial of weekly intravenous (iv) administration for 6 weeks with dose modifications at the highest dose investigated.

MATERIALS AND METHODS

Patients

Patients with histologically confirmed cancer not amenable to conventional treatment were eligible for inclusion in the study provided their renal function was adequate as determined by a blood urea nitrogen (BUN)

[1] Received May 2, 1974; accepted June 21, 1974.

[2] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text. NSC-141540 was supplied by Sandoz Ltd. (Hanover, NJ, and Basel, Switzerland) through Drug Research and Development, Division of Cancer Treatment, National Cancer Institute (NCI).

[3] NCI-VA Medical Oncology Branch, Veterans' Administration Hospital, Washington, DC.

[4] We would like to express our thanks to Mr. Frank Pelsor and Miss Mary Jo Bettis for their help in recording and collating the results of this study.

[5] *Reprint requests to*: Patrick J. Creaven, MD, NCI-VA Medical Oncology Branch, Veterans' Administration Hospital, 50 Irving St, NW, Washington, DC 20422.

level of less than 25 mg/100 ml and a serum creatinine level of less than 1.5 mg/100 ml. Other requirements included a serum bilirubin of less than 1.5 mg/100 ml, a serum glutamic oxaloacetic transaminase (SGOT) value of less than 100 IU, an absence of gross edema, pretreatment white blood cell (wbc) and platelet counts in excess of 5000 cells/mm^3 and 100,000 platelets/mm^3 respectively, and a sufficient interval after previous chemotherapy to avoid possible additive toxicity (2–8 weeks, depending on the preceding treatment).

The following tests were performed twice weekly during treatment and for at least 3 weeks after the completion of each treatment course: determination of wbc, differential, platelet, and reticulocyte counts; and determination of hematocrit. BUN, serum creatinine, SGOT, alkaline phosphatase, bilirubin, total serum proteins, serum albumin, serum calcium, and urinalysis were performed once weekly. Measurable tumors were measured by caliper using the product of the greatest horizontal and vertical diameters. Additional examinations such as skeletal and chest roentgenograms were obtained before the study. The chest roentgenograms were repeated every 2 weeks whenever measurable tumors were present; for patients without measurable tumors, roentgenographic examinations were repeated at the end of each study or when indicated clinically. The patients treated, their diagnoses, and their prior treatment are shown in table 1.

TABLE 1.—Patient information

Patient No.*	Age (yrs)	Diagnosis	Prior therapy†	Dose of NSC-141540 (mg/m^2)
1	56	Epidermoid carcinoma, lung	CCNU, PROCARB, STREP, BLEO, CYC	20
2	55	Epidermoid carcinoma, lung	CYC, MTX, CCNU, HYDROXY, STREP, BLEO	20
3	62	Adenocarcinoma, rectum	S, R, 5–FU, ICRF–159, ISOPHOS	20
4	63	Epidermoid carcinoma, head and neck	MTX–LCV, 5–FU	20
5	19	Acute lymphocytic leukemia	6-MP, VCR, MTX, PRED, L-ASP, CYC, DAUN	40
6	21	Acute lymphocytic leukemia	6-MP, VCR, MTX, PRED, ARA-C, L-ASP, DAUN	40
7	61	Anaplastic large cell carcinoma, lung	CCNU	40
8	54	Epidermoid carcinoma, lung	CYC, MTX	40
9	62	Clear cell carcinoma, kidney	ISOPHOS	70
10	45	Adenocarcinoma, primary site unknown	R	70
11	63	Adenocarcinoma, colon	S, 5-FU, CCNU, ISOPHOS	70
12	47	Adenocarcinoma, primary site unknown	5-FU	70
13	73	Adenocarcinoma, colon	5-FU	70
14	47	Epidermoid carcinoma, lung	—	100
15	49	Anaplastic carcinoma, primary site unknown	—	130
16	48	Epidermoid carcinoma, lung	R, THAL	130

TABLE 1.—Patient information—Continued

Patient No.*	Age (yrs)	Diagnosis	Prior therapy†	Dose of NSC-141540 (mg/m²)
17	49	Clear cell carcinoma, kidney	ICRF-159	130
18	82	Adenocarcinoma, stomach	S	170
19	43	Clear cell carcinoma, kidney	—	170
20	50	Adenocarcinoma, rectum	5-FU	170
21	44	Epidermoid carcinoma, lung	—	170
22	65	Epidermoid carcinoma, esophagus	R, ICRF-159	170
23	60	Epidermoid carcinoma, lung	R, CYC	170
24	52	Epidermoid carcinoma, head and neck	R, MTX	170
25	40	Epidermoid carcinoma, lung	CYC, MTX, VCR, BLEO	220
26	62	Small cell carcinoma, lung	R, CYC, MTX, CCNU, HYDROXY, VCR, PROCARB	220
27	63	Epidermoid carcinoma, lung	—	220
28	51	Adenocarcinoma, primary site unknown	—	220
29	55	Adenocarcinoma, lung	—	220
30	51	Epidermoid carcinoma, lung	—	220
31	57	Transitional cell carcinoma, bladder	R	220
32	61	Epidermoid cancer, lung	—	290
33	54	Small cell carcinoma, lung	R	290
34	50	Epidermoid carcinoma, lung	R	290
35	54	Adenocarcinoma, stomach	—	290
36	51	Small cell carcinoma, lung	CYC, MTX, HYDROXY, CCNU, VCR, PROCARB	290
37	61	Small cell carcinoma, lung	CYC, MTX, CCNU	290
38	57	Anaplastic large cell carcinoma, lung	R, ICRF-159, CCNU	290
39	44	Epidermoid carcinoma, lung	—	290
40	41	Adenocarcinoma, primary site unknown	—	290

*All patients except patient No. 6 were male.

†S = surgery, R = radiotherapy, CYC = cyclophosphamide, MTX = methotrexate, HYDROXY = hydroxyurea, VCR = vincristine, ISOPHOS = isophosphamide, STREP = streptozotocin, BLEO = bleomycin, LCV = leucovorin, ARA-C = cytosine arabinoside, PROCARB = procarbazine, PRED = prednisone, L-ASP = L-asparaginase, DAUN = daunorubicin, THAL = thalicarpine.

Drug Administration

The drug was supplied in ampules containing 100 mg of NSC-141540, 80 mg of Tween-80, 650 mg of polyethylene glycol 300, 30 mg of benzyl alcohol, 2 mg of anhydrous citric acid, and 1 mg of absolute alcohol. It was stored at room temperature. The contents of the ampules were added to 500 ml of 5% dextrose in water immediately before the beginning of a 1-hour constant iv infusion. Pulse and blood pressure were monitored at 15-minute intervals during the period of infusion.

Dose Modification

The course of treatment was 6 weeks. At doses up to and including 220 mg/m², treatment was omitted in the presence of leukopenia (wbc <5000 cells/mm³) or thrombocytopenia (platelet count <100,000 platelets/mm³). At the 290-mg/m² dose level, the following dose modification scheme was used: in the presence of wbc counts below 5000 but above 3000 cells/mm³ two thirds of the initial dose was given, and in the presence of wbc counts below 3000 cells/mm³ the dose was omitted. With platelet counts above 100,000 platelets/mm³ the full dose was given; with platelet counts below 100,000 but above 75,000 platelets/mm³ a two-thirds dose was given; and with platelet counts below 75,000 platelets/mm³ the dose was omitted. Patients were followed for 3 weeks after the end of treatment. A detailed study protocol is available upon request.

RESULTS

Forty patients each received a course of NSC-141540 at dose levels between 20 and 290 mg/m² of body surface area. Dose-limiting hematologic toxicity was seen consistently at doses of 170, 220, and 290 mg/m². Dose-limiting hematologic toxicity was not seen in any of the three patients treated with 130 mg/m² and was seen only erratically at doses below this level; the 17 patients treated with doses between 20 and 130 mg/m² will, therefore, be considered together. Leukopenia (wbc < 5000 cells/mm³) was seen in four patients, two of whom received 20 mg/m² and two of whom received 100 mg/m². The lowest wbc count (2600 cells/mm³) was seen in a patient (No. 3, table 1) who had had extensive radiotherapy to the pelvis and prior chemotherapy. Thrombocytopenia (platelet count 90,000 platelets/mm³) was seen in one patient who received a dose of 70 mg/m². Vomiting necessitating the interruption of drug administration occurred in two patients, both of whom received 130 mg/m². One patient (No. 2, table 1) had hypotension with a fall in blood pressure of 20–40 mm Hg after each drug administration. This patient had hypercalcemia. One patient (No. 12, table 1) who received 70 mg/m² had bradycardia which necessitated slowing of the drug administration; this patient was subsequently found to have pericardial involvement with tumor. Leukopenia (wbc < 5000 cells/mm³) was seen in all patients treated at doses of 170, 220, and 290 mg/m². The hematologic toxic effects noted at these doses are shown in table 2. It is clear that the toxic effects at doses of 170 and 220 mg/m² are essentially identical; the only substantial difference was that at 220 mg/m² one patient had marked leukopenia (wbc 600 cells/mm³) and thrombocytopenia. Nine patients were treated with 290 mg/m² in a phase I/phase II fashion with dose modification according to wbc or platelet count as indicated under Materials and Methods. This dose was more markedly toxic. Six of the nine patients had a wbc count of less than 3000 cells/mm³ (table 2); four of the nine patients were leukopenic within 1 week of the first dose so that the second dose had to be modified. The nadir of the wbc count was less than 3000 cells/mm³, and two of the nine patients had thrombocytopenia. A detailed analysis of the time-course of hematologic toxicity and the extent of the required dose modification is shown in figure 1. The mean number of doses received out of a maximum of six doses was 4⅔, ranging from 3⅔ to 5⅔ doses. Some nausea and occasional vomiting were seen in most patients treated at the three highest doses; these effects were not severe, were easily controlled by antiemetics, and did not require dose modifications. No other toxicity was seen in the 23 patients treated at the three highest doses; neither hypotension nor arrhythmia was observed during or immediately after drug infusion and there were no other signs of acute or chronic toxicity from the drug. No objective tumor responses were seen though it should be pointed out that most of the patients had extensive and far-advanced disease and had had extensive prior therapy. Six patients continued to receive the drug beyond the prescribed 6-week treatment period because of stable disease (patient Nos. 34, 37, 38, 39, and 40) or symptomatic improvement (patient No. 31). Symptomatic improvement consisted of relief of severe pain and improvement in performance status from 4 to 1 or 2. All the effects were short lived (< 20 weeks).

TABLE 2.—Hematologic toxicity from NSC-141540 at doses of 170–290 mg/m²

Dose (mg/m²)	No. of patients treated	No. of patients with wbc counts—(cells/mm³)				No. of weeks to leukopenia		Wbc nadir (cells/mm³)		No. of patients with platelet counts—(cells/mm³)		
		<5000	3000–4900	1000–2900	<1000	Median	Range	Median	Range	>100,000	99,000–75,000	<75,000
170	7	0	4	3	0	2	1–3	3150	2200–4100	7	0	0
220	7	0	4	2	1	2	1–3	3050	600–4900	6	1	0
290	9	0	3	5	1	1.5	1–3	2770	500–4000	7	1	1

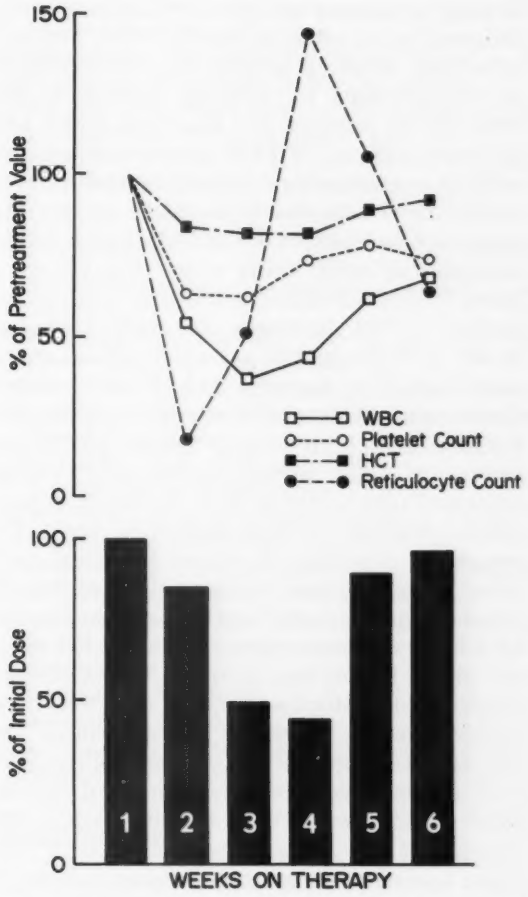


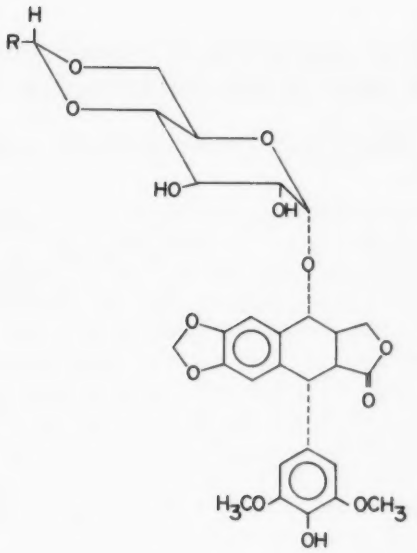
FIGURE 1.—Upper panel shows the mean response (observations in 9 patients) of the wbc count, platelet count, hematocrit level, and reticulocyte count to the 290-mg/m² dose of NSC-141540. Lower panel shows the mean percentage of initial drug dose given during each week of therapy to the same 9 patients using the dose modification described under Materials and Methods.

DISCUSSION

NSC-141540 is one of a series of podophyllotoxin derivatives which have shown activity against L1210 leukemia (4). The drug is closely related chemically to NSC-122819 which has previously been studied in this unit (fig 2) (2). However, in animal screening tests NSC-141540 was more active than the older com-

pound and showed an effect against intracerebrally inoculated L1210 leukemia in mice (4). It acts by causing an initial metaphase arrest followed by an action in late G_2 which prevents cells from entering mitosis (4). An inhibition of incorporation of tritiated thymidine into DNA in cell cultures has also been noted (4). In tissue cultures of P815 mouse mastocytoma cells the concentration required to produce 50% inhibition is reported to be 0.046 μg/ml. The compound is schedule dependent against L1210 leukemia in mice, being most effective on a twice weekly schedule. In addition to its action against L1210 leukemia, the compound has shown activity against a variety of other tumors including Sarcoma 180, Ehrlich ascites tumor, and Walker 256 carcinosarcoma (4). Because of its high activity against L1210 leukemia and other animal tumors and its demonstrated superiority to NSC-122819 in experimental systems, it was decided to study the compound clinically. A phase I clinical trial using daily × 7 and twice weekly for 3-week schedules had already been initiated in Europe by Nissen and co-workers (3) when the present phase I trial was initiated. We therefore decided to investigate tolerance to a weekly × 6 dose schedule. Tolerance to single doses was not studied because of the possibility that with single large doses the toxicity of the vehicle might become dose limiting before the toxicity of the drug. When we started our phase I trial, 30 mg/m² twice weekly seemed to be the moderately toxic dose in man as determined by Nissen's study. We therefore decided to initiate our clinical trial at one third of this dose on a weekly basis (20 mg/m²/week) because it seemed likely that weekly doses would be no more toxic than the same dose given in divided portions. We escalated the dose according to the modified Fibonacci search previously described and found safe for initial clinical investigation of anticancer agents (5). The results obtained (table 2, fig 1) confirmed the findings of Nissen et al (3) that (*a*) hematologic toxicity is essentially the only dose-limiting toxic effect found in patients well controlled with antiemetics, (*b*) the compound is relatively platelet sparing, and (*c*) there is some decrease in the hematocrit level in some patients during the course of treatment. The results also confirm our impression with other drugs that the reticulocyte count is probably the most sensitive early indicator of hematologic toxicity (fig 1). The present results indicate that NSC-141540 is not markedly schedule dependent when comparing a twice weekly and a once weekly schedule of administration, the hematologic toxicity found at 170 mg/m² in the present study being essentially equivalent to that found by Nissen et al with a dose of 86 mg/m² twice weekly (172 mg/m²/wk) (3). It is also clear from the present results that the drug has a shallow dose-response curve; it is possible to increase a consistently toxic dose by a factor of almost two and remain within a relatively safe dosage range. However, in the present study, it should be noted that many of the patients treated at the lower dose had received extensive prior chemotherapy and/or radiotherapy whereas a substantial number of the patients treated at the highest dose had received no prior treatment (table 1). This could account, in part, for the fact that mild but definite hematologic toxicity was seen at a dose level less than one tenth of the moderately toxic dose finally achieved.

As noted above, one of the features of the present study design was the omission of an investigation of single-dose administration due to a fear that the results would be invalidated by vehicle toxicity. However, it is clear that a dose level was reached on the weekly administration schedule which was close to the moderately toxic dose that would have been reached if the drug had been given as a single dose, since four of nine patients treated at the highest dose already had toxic reactions when the second dose was given. Four of the remaining five patients had toxic reactions within 3 days after the second dose, and it is quite possible that the toxicity would have occurred at this time even if the second dose had not been given. It is probable, therefore, that our more usual study design in which we investigate the toxicity of single doses before investigating a schedule of administration would have been both safe and feasible in the present case. In addition, had the toxicity of a single dose been



I. R =

II. R = CH_3–

FIGURE 2.—Structures of NSC-122819 (I) and NSC-141540 (II).

established at or near 290 mg/m², the weekly schedule of administration could have been started at one third of this dose and the study could thereby have been considerably shortened. The results of this study confirm that NSC-141540 is a drug with almost exclusively hematologic toxicity. It can be safely administered on a weekly schedule at a starting dose of 290 mg/m² with suitable dose modification, except in patients who have had extensive prior chemotherapy and/or radiotherapy. This dose and schedule can be recommended for phase II clinical trials.

LIST OF COMPOUNDS

L-Asparaginase: NSC-109229; CAS reg. No. 9015-68-3

Bleomycin: NSC-125066

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride

Daunorubicin: NSC-83142; CAS reg. No. 23541-50-6; daunomycin (NSC-82151)

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8

Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1

ICRF-159: NSC-129943; CAS reg. No. 21416-87-5; 2,6-piperazinedione, 4,4′-propylenedi-, (±)-

Isophosphamide: NSC-109724; CAS reg. No. 3778-73-2; 1,3,2-oxazaphosphorine, 3-(2-chloroethyl)-2-[(2-chloroethyl)amino]tetrahydro-, 2-oxide

Leucovorin: NSC-3590; CAS reg. No. 6035-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]-, calcium salt

6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate; 6-MP

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-

NSC-122819: 4′-demethylepipodophyllotoxin 9-(4,6-*O*-2-thenylidene-β-D-glucopyranoside); VM-26

NSC-141540: 4′-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside); VP-16-213

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride

Streptozotocin: NSC-85998

Thalicarpine: NSC-68075

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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Phosphoribosyl Transferase in Colon Tumor and Normal Mucosa as an Aid in Adjuvant Chemotherapy With 5-Fluorouracil (NSC-19893) [1,2,3]

Aly Nahas,[4] Edwin D. Savlov,[4] and Thomas C. Hall [4,5,6,7]

5-Fluorouracil (5-FU) has proved to be effective in the palliation of patients with inoperable carcinomas of the colon and rectum (1). It elicits objective responses in approximately 20% of the patients treated, and those who respond have a mean survival time twice that of nonresponders or untreated patients.

In the case of operable tumors, the use of 5-FU as an adjuvant to surgery is theoretically attractive. But since only 20% of patients with colon carcinomas respond to systemic 5-FU, one can try to improve adjuvant chemotherapy by preselecting patients with responsive tumors and then treating with 5-FU only those patients most likely to respond. We approached this problem by looking at the activities of enzymes involved in the incorporation of 5-FU into the cell metabolic system.

Accumulated evidence (2) suggests that the metabolism of 5-FU parallels that of uracil. 5-FU is converted to its nucleotide, fluorouridine monophosphate (FUMP), via a uracil phosphoribosyl transferase (PRT). FUMP is then reduced to fluorodeoxyuridine monophosphate (FdUMP) which competes with deoxyuridine monophosphate (dUMP) for the thymidylate synthetase (TS), thereby preventing thymidylic acid synthesis. It has been shown in our laboratory that the prevention of such synthesis leads to a decreased level of the thymidylate triphosphate (dTTP), and that such decrease may induce the activity of thymidine kinase (TK). TK, however, serves two opposite purposes: it may lead to biosynthesis of more FdUMP, but it can also catalyze the conversion of thymidine to thymidylic acid in a salvage pathway, thus bypassing the FdUMP block. In theory, to be responsive to 5-FU, the ideal situation would be for a tumor to have a low TK level but a high PRT level, assuming that other mechanisms of resistance are absent. Reyes and Hall (3) submitted evidence supporting this reasoning by demonstrating that the sensitivity to 5-FU by various mouse tumors was directly correlated to tumor PRT levels. On the other hand, Kessel and Wodinsky (4) have shown that this sensitivity is inversely proportional to tumor TK levels.

METHODS

Patient Selection and Clinical Evaluation

Tumor tissue and adjacent normal mucosa are obtained at the time of surgery from patients with histologically verified colon carcinomas. Tissues are then assayed for the enzymes and the data are stored. When recurrence of the carcinoma is confirmed by physical examination, chest roentgenograms, liver biopsy, liver scan, or other means, the patient is treated with 5-FU, 15 mg/kg/day for 5 days given orally followed by 15 mg/kg/week. A lower dose of 12 mg is used in patients who have received radiation therapy or who are debilitated. A positive response is defined as at least a 50% reduction in the size of the

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[2] Supported by Public Health Service grant CA-11198 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] 5-Fluorouracil: CAS reg. No. 51-21-8; 5-FU. Supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, NCI.

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[6] The authors wish to acknowledge the useful discussions with Dr. Bruce Hacker, Division of Oncology, Albany Medical Center, NY.

[7] *Reprint requests to:* Dr. Aly Nahas, Department of Biochemistry, University of Rochester Medical Center, Rochester, NY 14642.

measurable lesions within 3–4 weeks lasting at least 2 months. When comparing enzyme activities with clinical response to 5-FU, only patients who have not received other therapy between the time of surgery and the time of recurrence are entered into our evaluation.

Enzyme Assays

The malignant or normal tissue is pulverized in liquid nitrogen and the 100,000 × g cytosol is prepared. In brief, TS is assayed by the appearance of tritiated water in the methylation of deoxyuridine-5′-monophosphate-5-^{3}H where the remaining dUMP-^{3}H is removed by adsorption on charcoal (5). Thymidine-2-^{14}C is the substrate for TK (6), and 5-FU-2-^{14}C and phosphoribosyl pyrophosphate are the substrates for PRT (3). In either case, nucleotide products remain at the origin during chromatography on anion exchange paper and can be counted. Data are computed as nmol/g protein/hr.

RESULTS AND DISCUSSION

Over the past year, approximately 100 matched tissue pairs (tumor and adjacent normal mucosa) from patients with colonic carcinoma have been tested for TS, TK, and PRT. As shown in table 1, there is a wide variation in the TS activity in different patients regardless of the type of tissue. Enzyme activity varied between less than 1 and 1400 nmol/g protein/hr. There is no significant difference in the distribution of the TS activity between normal and tumor tissues. In the case of TK, 12% of the tumors had activities greater than 1000 nmol/g protein/hr while only 3% of normal tissues showed similar values. In the case of PRT, a similar analysis shows that 25% of the assayed carcinomas had activities greater than 1000 nmol/g protein/hr as compared to 3% of the normal tissues having comparable values. Furthermore, the enzyme data on individual patients could not be correlated with their responsiveness to 5-FU. However, by computing the ratio of the tumor enzyme level to that of the normal tissue enzyme level in each patient and comparing these ratios to responsiveness to 5-FU, a different picture emerges (table 2). This ratio is 1 or less in approximately 50% of the patients with respect to TS or PRT but in only 27% of the patients with respect to TK. The ratio is slightly higher than 1 in 24% and 13% of the patients for TS and PRT respectively but in 53% of the patients for TK. Looking at it another way, the ratio is 5 or less in 95% of the patients for TK and in 73% of the patients for PRT. However, higher ratios occur more often for PRT than for the other two enzymes. Of the three enzymes studied a ratio of 50 is exceeded only in the case of PRT (one out of every seven patients studied).

The number of patients who have had recurrence and have been treated with 5-FU by our criteria is still limited. It is interesting to note, however, that among four patients who had a positive clinical response, tumor tissue/normal tissue enzyme activity ratios (T/N) for PRT were 8 or more in three patients (table 3), while in the four nonresponders T/N ratios for the same enzyme were less than 1 in three patients. It is clear from table 3 that enzyme values from individual patients bear no relationship to clinical response.

In several of the patients who were unresponsive to 5-FU, tumor PRT values were higher than in some of the responders. These preliminary data suggest that the ratio of PRT in tumors versus PRT in normal mucosa may be used to predict responsiveness of patients with colon carcinomas to adjuvant therapy with 5-FU. A possible explanation is that this ratio may determine the distribution of 5-FU between tumor and normal tissues. A high ratio may very well lead to high concentrations of 5-FU and its active metabolites in the tumor. This possibility is consistent with the observation by Rutman et al (7) in 1954 that uracil is utilized more by rat hepatoma than by normal liver and with Heidelberger's later report (8) that colon carcinoma converted more 5-FU to nucleotide than did normal colon epithelium.

Failure of patients with high T/N ratios for PRT to respond to 5-FU (as in the case of patient HH 487 in table 3) can perhaps be explained in terms of changes in other metabolic pathways, such as elevated TK levels. Un-

TABLE 1.—Enzyme activities in human colon

nmol/g protein/hr	Percent of total*					
	TS		TK		PRT	
	N	T	N	T	N	T
<1	3	8	0	1	44	39
1–5	1	3	0	0	0	1
6–10	5	5	1	0	0	0
11–20	7	5	0	0	1	3
21–50	19	23	11	4	10	6
51–100	23	19	22	16	14	7
101–200	17	19	20	23	11	3
201–1000	18	21	43	44	19	17
1001–2000	0	0	3	12	1	9
>2000	0	0	0	0	2	16

*N = normal tissue; T = tumor tissue.

TABLE 2.—Enzyme activities in human colon tumor tissue vs normal tissue

T/N*	Percent of total		
	TS	TK	PRT
<1	46	20	20
≃1	3	7	33
1.1–2	24	53	13
2.1–5	12	15	7
5.1–10	7	3	8
10.1–20	0	1	2
20.1–50	8	1	4
>50	0	0	14

$$*T/N = \frac{\text{enzyme activity in tumor tissue}}{\text{enzyme activity in normal tissue}}.$$

TABLE 3.—PRT activity and clinical response to 5-FU

	nmol/g protein/hr*		
	N	T	T/N†
Responders			
HH 363	10	1990	199
HH 365	0.3	20	67
SMH 600	100	800	8
HH 188	90	24	0.3
Nonresponders			
HH 487	125	1060	8.5
HH 230	174	155	0.9
HH 284	690	355	0.7
SMH 365	10	7	0.7

* N = normal tissue; T = tumor tissue.

$$\dagger T/N = \frac{\text{enzyme activity in tumor tissue}}{\text{enzyme activity in normal tissue}}.$$

fortunately, we do not have the TK values for this patient to make a firm conclusion.

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Randomized Study Comparing CCNU (NSC-79037) and Methyl-CCNU (NSC-95441) in Advanced Bronchogenic Carcinoma [1,2,3]

Robert T. Eagan, David T. Carr, Douglas T. Coles, David E. Dines, and Roy E. Ritts Jr.[4,5]

SUMMARY

One hundred and twenty-four patients with advanced lung cancer were entered into a randomized study comparing CCNU and methyl-CCNU. The overall regression rate at the time of the first followup visit after treatment had started was 3.2% (two of 63 patients) with CCNU and 6.6% (four of 61 patients) with methyl-CCNU. The response rates for CCNU and methyl-CCNU by cell type were as follows: epidermoid—5.6% for both drugs; adenocarcinoma—no responses with either drug; large-cell—no responses with CCNU and 8.3% (one of 12 patients) with methyl-CCNU; small-cell—12.5% (one of eight patients) with CCNU and 25.0% (two of eight patients) with methyl-CCNU. If both objective regressions and stable disease were added together, the "response" rates were 36.5% for CCNU and 36.1% for methyl-CCNU. The presence or absence of skin-test delayed-type hypersensitivity made no difference in response rates. Patients classified as having regression or stability of disease had a significantly better survival rate than did patients whose disease progressed.

[Cancer Chemother Rep 58:913–918, 1974]

CCNU and methyl-CCNU are nitrosourea derivatives with antitumor activity in a variety of animal and human tumors. Both agents are highly lipid soluble. Their carcinostatic activity may reflect a dual action of modifying cellular proteins by cyclohexylcarbamoylation and nucleic acids by alkylation (1).

CCNU is active against mouse L1210 leukemia and mouse Lewis lung tumors.[6] Methyl-CCNU was found to be superior to CCNU in the advanced Lewis lung tumor in mice where the former effects cures.[6]

In his review of monochemotherapy of bronchogenic carcinoma, Selawry (2) found the nitrosourea compounds to be among the more active agents tested, with CCNU being ranked first in epidermoid bronchogenic tumors and 1-methyl-1-nitrosourea (NSC-23909) first in the small-cell type.

[1] Received May 15, 1974; revised Aug 12, 1974; accepted Aug 21, 1974.

[2] Supported in part by contract N01-CM-33735 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] CCNU: CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-.

Methyl-CCNU: CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-.

Both drugs were supplied by Cancer Therapy Evaluation, DCT, NCI.

[4] Medical Oncology, Mayo Clinic, Rochester, Minn.

[5] *Reprint requests to:* Robert T. Eagan, MD, Medical Oncology, Mayo Clinic, Rochester, Minn 55901.

[6] Broder LE, and Carter SK. Clinical brochure: 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (Me-CCNU; NSC-95441). Prepared by the NCI, Bethesda, Md, Nov 1971.

Takita and Brugarolas (3) reported a 36% response rate for CCNU in bronchogenic carcinomas. In addition, they felt that the presence or absence of skin-test delayed-type hypersensitivity (DTH) to recall microbial antigens was a critical determinant of response. In their patients with absent DTH, the response rate was only 5.6% versus 70.6% in patients with a skin-test DTH reaction greater than 1 cm in diameter.

This study was designed to compare the efficacy of CCNU and methyl-CCNU in the four major cell types of bronchogenic carcinoma as defined by the World Health Organization (4). To make the comparison as fair as possible, all patients were stratified by cell type, skin-test DTH status, and performance score (PS) before randomization.

MATERIALS AND METHODS

As of March 31, 1974, when the study was terminated, 124 patients had been entered. Of these patients, 119 returned for at least one reevaluation. Five patients did not return after the initial treatment and were considered treatment failures. There were 87 men and 37 women. The median age of the men was 60 years (range 41–78 years).

Before patients were randomized between CCNU and methyl-CCNU treatment, they were stratified by cell type, skin-test DTH, and PS (Eastern Cooperative Oncology Group scale). The cell types used were the four major types in the World Health Organization classification (4): epidermoid, small-cell anaplastic, adenocarcinoma, and large-cell. The skin tests employed to assess DTH included purified protein derivative, 5 toxic units (liquid, Tween-stabilized, Connaught Laboratories Ltd.); mumps, 2 colony-forming units (Eli Lilly and Company); streptokinase-streptodornase, 50 units of streptokinase (Varidase, Lederle Laboratories); *Candida*, 1:100 extract of *Candida albicans* (Hollister-Steir); and a phosphate-buffered saline control. Dinitrochlorobenzene was also used. The sensitizing dose given on the patient's initial visit was 2 mg in acetone. Dinitrochlorobenzene challenge skin tests of 0.2, 0.1, 0.05, and 0.25 mg (Ortho Pharmaceutical Company) were given on return visits and scored using the method of Catalona et al (5).

Skin tests were read at 48–72 hours. All recall skin tests were recorded as millimeters of induration in two diameters less any reactivity of the phosphate-buffered saline control site. For subsequent analysis and for comparison with Takita and Brugarolas' results (3), reactions 1 cm or greater were considered positive and those less than 1.0 cm were considered negative. The Eastern Cooperative Oncology Group PS scale of 0–4 was used. Patients with PSs of 0–2 were arbitrarily classified as "good" and those with scores of 3 or 4 were classified as "bad."

After this stratification, patients were randomized between CCNU and methyl-CCNU. CCNU was given in a dose of 130 mg/m^2 orally every 7 weeks. The initial dose of methyl-CCNU was 200 mg/m^2 orally. Subsequent doses beginning 7 weeks later were 150 mg/m^2 orally every 7 weeks. There was no upward dose escalation. Subsequent doses were decreased by one third if the white blood cell (wbc) nadir was less than 2000 cells/mm^3 or the platelet nadir was less than 75,000 platelets/mm^3.

Three categories of response were used: regression, stable, and progression. A regression required a 50% or greater reduction in the product of the two largest perpendicular diameters of the indicator lesion with no new lesions appearing and no progression of any other lesions. Patients' diseases were considered stable if there was less than a 50% reduction or less than a 25% increase in the indicator lesion with all other lesions being stable and no new lesions appearing. Progressions required a 25% or greater increase in the size of the indicator lesion or the appearance of new lesions. All patients were evaluated for response on an every-7-week schedule.

Sixteen of the 124 patients had had prior chemotherapy.

RESULTS

In all patients manifesting regression of disease, the regression occurred after the initial course of treatment. No patient classified as stable on evaluation after the first course of treatment ever achieved objective regression status.

No objective regressions were noted in the 16 patients who had had prior chemotherapy. Of these 16 patients, six had received mechlorethamine, five had received 5-fluorouracil, three had received cyclophosphamide, one had received methotrexate, and one had received both 5-fluorouracil and cyclophosphamide.[7] If only previously untreated patients are considered, the response rate for CCNU was 3.5% and for methyl-CCNU 7.8%. The overall response rates, all patients included, were 3.2% for CCNU and 6.6% for methyl-CCNU (table 1). Combining the regressions with the stable diseases, the results were 36.5% and 36.1% respectively.

The response rates for patients treated with CCNU and methyl-CCNU according to the four major cell types previously described are presented in table 2. There were 36 cases of epidermoid-cell type. Eighteen patients were treated with CCNU with 5.6% of these diseases regressing and 38.9% remaining stable. Of the 18 patients treated with methyl-CCNU, 5.6% had disease regression and 50.0% had disease stability. There were 47 cases of adenocarcinoma-cell type with no regressions noted with either agent in this group. Stability of disease was observed in 45.8% of patients treated with CCNU and in 26.1% who were treated with methyl-CCNU. Twenty-five patients with the large-cell type were treated. No disease regressions were seen with CCNU and only 15.4% of these patients had disease that remained stable. One disease regression (8.3%) and two patients with disease stability (16.7%) were noted after treatment with methyl-CCNU. Sixteen patients with the small-cell type were treated. One of eight responded to CCNU and two of eight responded to methyl-CCNU. Only one patient on each drug remained in the stability of disease status.

Median survival times are shown in table 3. Survivals were calculated from the time of entry on study to the time of last followup or death. The median survival time of patients with the epidermoid- and adenocarcinoma-cell types was 19 weeks compared to 9 weeks for patients with the small-cell type. Analysis of the death rates for these three cell types revealed that the death rate for patients with the small-cell type was significantly higher ($P < 0.05$) than the death rates for patients with either the epidermoid or adenocarcinoma types.

[7] Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride; HN2; nitrogen mustard.

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU.

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan.

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-.

TABLE 1.—Response rates for CCNU and methyl-CCNU with all cell types grouped together for patients with and without prior chemotherapy

Treatment	Total No. of patients	No. / % of patients with—		
		Regression	Stable disease	Progression
CCNU				
Prior chemotherapy	6	0/0.0	2/33.3	4/66.7
No prior chemotherapy	57	2/3.5	19/33.3	36/63.2
Total	63	2/3.2	21/33.3	40/63.5
Methyl-CCNU				
Prior chemotherapy	10	0/0.0	2/20.0	8/80.0
No prior chemotherapy	51	4/7.8	16/31.4	31/60.8
Total	61	4/6.6	18/29.5	39/63.9
Total CCNU and methyl-CCNU	124	6/4.8	39/31.4	79/63.7

TABLE 2.—Response rates for CCNU and methyl-CCNU by cell type

Treatment	Total No. of patients	No. / % of patients with— Regression	Stable disease	Progression
		Epidermoid		
CCNU	18	1/5.6	7/38.9	10/55.6
Methyl-CCNU	18	1/5.6	9/50.0	8/44.4
Totals	36	2/5.6	10/44.4	18/50.0
		Adenocarcinoma		
CCNU	24	0/0.0	11/45.8	13/54.2
Methyl-CCNU	23	0/0.0	6/26.1	17/73.9
Totals	47	0/0.0	17/36.2	30/63.8
		Large-cell carcinoma		
CCNU	13	0/0.0	2/15.4	11/84.6
Methyl-CCNU	12	1/8.3	2/16.7	9/75.0
Totals	25	1/4.0	4/16.0	20/80.0
		Small-cell carcinoma		
CCNU	8	1/12.5	1/12.5	6/75.0
Methyl-CCNU	8	2/25.0	1/12.5	5/62.5
Totals	16	3/18.8	2/12.5	11/68.8

Patients with "positive" DTH ($\geq$ 10 mm) and "good" PSs did better than patients with "negative" DTH ($<$ 10 mm) and "bad" PSs (table 4). However, the majority of patients (60.8%) with "good" PSs had positive DTH while the majority of patients with "bad" PSs (71.9%) had negative DTH using the 10-mm criteria. Nineteen patients had skin recall reactivities of 4–9 mm and so were not actually anergic. However, this did not affect the above analysis. A detailed study of several measures of cellular-mediated immunity in advanced lung cancer is in preparation. Patients with disease regressions lived significantly longer than did patients with either stability or progression of disease.

TOXICITY

Toxicity was comparable for CCNU and methyl-CCNU (table 5). No unusual side effects were noted. There were no drug-related deaths. Wbc and platelet counts were obtained at least once a week for 6 weeks. The majority of patients treated with both agents had some degree of hematologic toxic effects. The degree of this toxicity, or whether or not it occurred at all, did not seem to influence response rates.

DISCUSSION

The regression rates noted with both CCNU and methyl-CCNU were quite disappointing and considerably below the response rates in

TABLE 3.—Effects of various parameters on duration of median survival

Parameter	Total No. of patients	Median survival (wks)
CCNU	63	16
Methyl-CCNU	61	17
PS		
Good	91	19
Bad	33	10
DTH		
Positive	65	19
Negative	59	14
Epidermoid	36	19
Adenocarcinoma	47	19
Large-cell	25	14
Small-cell	16	9
Regression	6	54
Stable	39	27
Progression	79	10

TABLE 4.—Response rates for CCNU and methyl-CCNU based on PS and the presence or absence of skin-test DTH

Treatment	DTH	PS	Total No. of patients	No. /% of patients with— Regression	Stable disease	Progression
CCNU	Positive	Good	29	1/3.4	11/37.9	17/58.6
		Bad	5	0/0.0	1/20.0	4/80.0
	Negative	Good	18	0/0.0	7/38.9	11/61.1
		Bad	11	1/9.1	2/18.2	8/72.7
Total CCNU			63	2/3.2	21/33.3	40/63.5
Methyl-CCNU	Positive	Good	27	2/7.4	8/29.6	17/63.0
		Bad	4	0/0.0	3/75.0	1/25.0
	Negative	Good	18	1/5.6	5/27.8	12/67.7
		Bad	12	1/8.3	2/16.7	9/75.0
Total methyl-CCNU			61	4/6.6	18/29.5	39/63.9

TABLE 5.—Comparison of toxicity between CCNU and methyl-CCNU in 119 of 124 patients on whom data were available

Toxicity	CCNU*	Methyl-CCNU*
Nausea	31/59	26/60
Vomiting	26/59	24/60
Diarrhea	11/59	4/60
Anorexia	14/59	10/60
Jaundice	0/59	0/60
Wbc nadir†		
3000–3999	15/59	8/60
2000–2999	11/59	16/60
1000–1999	10/59	6/60
<1000	1/59	2/60
Platelet nadir†		
75,000–99,999	7/59	9/60
50,000–74,999	6/59	10/60
25,000–49,999	8/59	5/60
<25,000	5/59	4/60

*Total No. of patients with toxic reaction/total No. of patients treated.

†Cells/mm³.

the Selawry report (2). One possible reason for the incompatibility in response rates might be differences in what criteria must be satisfied before a patient is said to have had a response. Using the response criteria of Takita and Brugarolas (3), ie, complete responses, partial responses, and stability of disease added together, our "response" rate for CCNU would be 36.2%. This response rate would be essentially identical to that reported in their article.

We feel that our response criteria are justified for two reasons. First, patients in this study having less than a 50% reduction in tumor size after the first treatment always eventually had disease progression. Second, if anything less than a 50% reduction in tumor size is used for a response, and especially if stability of disease is used, individual differences in tumor growth rates might be considerably more important than malignant cell kill by the drugs. This is particularly true when considering prolongations in survival.

The severity of hematologic depression, at least with CCNU, seemed comparable to other reports. Takita and Brugarolas (3) reported that 20% of their patients had a wbc nadir

of 3000 cells/mm³ or lower, with only 6% having a nadir of 2000 cells/mm³ or lower. We found 37% of our patients had a wbc nadir less than 3000 cells/mm³ and 18% had a nadir less than 2000 cells/mm³. Twenty-four percent of their patients had a platelet nadir less than 80,000 platelets/mm³ as compared to 30% of our patients with a platelet nadir less than 75,000 platelets/mm³.

The presence or absence of skin-test DTH did not influence the response rates with either agent studied in contrast to the report of Takita and Brugarolas (3). We can not account for this discrepancy since our skin-testing techniques and definition of "positivity" for analysis were quite similar. We recorded our recall skin-test responses as the mean induration in two dimensions rather than the greatest diameter. Although this information was not given in their report (3), in a later study (6) Takita and Brugarolas used the greatest diameter. This difference might have the effect of making our patient response as a group less reactive than theirs, but reanalysis of our data in these terms places only 12 of 19 patients with less than 1-cm responses into the greater than 1 cm reactivity category. This change did not affect the stated results.

We feel that CCNU and methyl-CCNU have no significant value as single agents in the chemotherapy of bronchogenic carcinoma.

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Adriamycin (NSC-123127) in Combination With Cyclophosphamide (NSC-26271): A Phase I and II Evaluation [1,2,3]

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SUMMARY

Combination therapy with adriamycin and cyclophosphamide was evaluated in 29 patients with various solid tumors. The drugs were administered concurrently intravenously every 3 weeks. The dose of cyclophosphamide was 500 mg/m² throughout the study. Adriamycin was initially given in doses of 25 mg/m² to the first three patients, and subsequently to 13 patients who had received extensive prior therapy. Another 13 patients received 37.5 mg/m² of adriamycin in combination with cyclophosphamide. One objective response was observed in a patient receiving the low-dose adriamycin combination, and three responses including one complete response were observed in patients receiving the high-dose adriamycin combination. Noteworthy were the results achieved in three patients with endometrial carcinoma, all of whom received the higher dose adriamycin-cyclophosphamide combination. All three of these patients benefited from this treatment; one complete remission and one partial remission were sustained for 9+ and 4+ months, respectively, and the third patient improved subjectively with no objective progression for 3 months. The major toxicity observed was leukopenia with the nadir occurring between Days 10 and 15. Alopecia was nearly universal. Nausea and vomiting did not constitute a major problem. Further studies of the combination of adriamycin (37.5 mg/m²) and cyclophosphamide (500 mg/m²) are recommended in the treatment of metastatic carcinoma of the corpus uteri, breast, lung, and ovary, and in soft tissue sarcomas. An evaluation of this combination's comparative efficacy relative to sequential drug regimens is needed to determine whether the combination possesses true therapeutic synergism.

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[3] Adriamycin and cyclophosphamide were supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, NCI. Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

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Adriamycin (1) and cyclophosphamide (2) are useful chemotherapeutic agents when used individually for the treatment of a variety of advanced cancers. The major dose-limiting toxicity for both drugs is hematopoietic depression affecting predominantly the white blood cell (wbc) series; both drugs cause reversible alopecia and gastrointestinal symptoms, predominantly nausea and vomiting. The drugs have commonly been used as single intravenous (iv) injections given every 3 weeks, and acceptable toxicity has been encountered after administering 60–75 mg/m^2 of adriamycin (1) and 1000–1500 mg/m^2 of cyclophosphamide (3), respectively. Because both drugs appear to have a similar spectrum of activity against a number of human neoplasms, a study of a combination of these agents on an every-3-week schedule was initiated. The purpose was to establish the tolerance to such a combination of adriamycin and cyclophosphamide using equivalent fractions of their tolerable doses when used alone. In addition, we hoped to make preliminary observations on the possible usefulness of this combination in the treatment of malignancies where both agents had been previously found to be effective.

PATIENTS AND METHODS

Twenty-nine adult patients with biopsy-proven metastatic malignancies were selected for treatment with adriamycin and cyclophosphamide. All patients had progressive disease which was either measurable or evaluable. None had received prior therapy with either agent or with other alkylating agents, with the exception of four patients: one with an adenocarcinoma of the breast, one with an adenocarcinoma of the parotid gland who had received courses of cyclophosphamide in daily oral schedules, and one each who had brief exposures to mechlorethamine and chlorambucil, respectively. The diagnoses, distribution of disease, and prior treatment are shown in tables 1 and 2.

Adriamycin was initially given at a dose level of 25 mg/m^2 combined with 500 mg/m^2 of cyclophosphamide (table 1). The subsequent dose level that was evaluated included an escalation to 37.5 mg/m^2 of adriamycin (table 2). A third dose level was planned in the event of minimal or no toxicity at the preceding step employing 37.5 mg/m^2 of adriamycin and 750 mg/m^2 of cyclophosphamide. The drugs were given by iv push every 3 weeks with phenothiazine antiemetics administered routinely. Patients were to receive a minimum of two courses before discontinuing treatment because of progressive disease. On the other hand, patients showing no change or objective responses were to continue receiving treatment until the total dose of adriamycin exceeded 550 mg/m^2 in order to avoid the cardiotoxicity associated with this drug. The status of their disease was assessed prior to each course by measurement of the product of the largest cross diameter of discrete lesions, including followup of other disease-related manifestations. A complete remission (CR) was defined as the complete disappearance of any evidence of disease, and a partial response (PR) was defined as a 50% or greater reduction in the size of measurable lesions. Changes that did not meet these criteria and were not accompanied by clinical deterioration were classified as no change (NC). If new lesions appeared or lesions increased in size by 25%, the study was discontinued and patients were considered to have progressive disease (PD).

Hematologic changes (including wbc and platelet counts), chemical determinations, and assessment of other drug-related toxicities were evaluated prior to each treatment and during the first two courses, at least once midway during the treatment. An electrocardiogram was obtained prior to treatment and at 3-month intervals thereafter.

The dose level was reduced by one third if leukopenia (wbc count $<$4000 cells/mm^3) and/or thrombocytopenia (platelet count $<$ 100,000 cells/mm^3) had occurred by Day 22. Treatment was postponed if the wbc count was $<$ 3000 cells/mm^3 or if the platelet count was $<$ 75,000 platelets/mm^3. The dose of cyclophosphamide was escalated by one third in patients failing to show any hematologic effect.

RESULTS

The results of therapy with the combination of adriamycin and cyclophosphamide at the

TABLE 1.—Treatment with adriamycin (25 mg/m²) and cyclophosphamide (500 mg/m²) every 3 weeks

Patient No.	Age/sex	Diagnosis, site	Site of metastases	Prior therapy*	No. of courses	Total dose (mg)		Response†
						Adriamycin	Cyclophosphamide	
1	47/F	Adenocarcinoma, breast	Liver, soft tissue, pleura	RT, oral cyclophosphamide + prednisone, 5-FU + MTX + VCR	3	100	2000	PD
2	41/F	Adenocarcinoma, breast	Bone, cord, soft tissue	Oophorectomy, RT, androgens, 5-FU, prednisone	2	75	1500	PD
3	71/F	Adenocarcinoma, breast	Liver, bone, lung, pleura, soft tissue	RT, estrogens, androgens	2	60	1100	PD
4	59/F	Adenocarcinoma, breast	Liver, pelvis, ascites	RT, 5-FU + VCR + prednisone	3	120	2400	PD
5	29/F	Adenocarcinoma, ovary	Peritoneum	RT, chlorambucil, 5-FU	5	150	3000	NC (3 mos); refused further therapy‡
6	54/F	Adenocarcinoma, ovary	Lung, pleura	Intrapleural HN_2	9	360	7275	PR (6 mos)
7	48/F	Adenoid cystic carcinoma, Bertholin gland	Liver, lung, soft tissue	RT, 5-FU, *d*-tetrandrine	4	120	2800	PD‡
8	52/M	Large cell undifferentiated, lung	Lung, pericardium, bone	RT	4	200	4000	NC (2 mos); PD
9	33/M	Large cell undifferentiated, lung	Lung, meninges, bone, soft tissue	None	1	40	850	PD; died on Day 17
10	50/F	Epidermoid carcinoma, cervix	Pelvis, supraclavicular lymph node	RT	1	30	600	NE; refused further therapy‡
11	84/F	Transitional cell carcinoma, bladder	Bladder	RT	1	39	800	NE; refused further therapy
12	64/M	Transitional cell carcinoma, bladder	Pelvis, inguinal lymph node	RT	3	108	2100	PD‡
13	69/F	Epidermoid carcinoma, bladder	Pelvis	RT	1	40	800	NE; died on Day 4‡
14	55/F	Mesodermal carcinoma, ovary	Peritoneum, ascites	None	2	80	1200	PD; adriamycin alone, 2 doses

TABLE 1.—Treatment with adriamycin (25 mg/m²) and cyclophosphamide (500 mg/m²) every 3 weeks—Continued

Patient No.	Age/ sex	Diagnosis, site	Site of metastases	Prior therapy*	No. of courses	Total dose (mg)		Response†
						Adriamycin	Cyclophosphamide	
15	73/F	Rhabdomyosarcoma, buttock	Bone, lungs, soft tissue	RT	2	75	1500	PD
16	64/M	Rhabdomyosarcoma,liver	Liver	None	1	45	900	PD; died on Day 16

*RT = radiotherapy; 5-FU = 5-fluorouracil; MTX = methotrexate; VCR = vincristine; HN_2 = mechlorethamine.
†PD = progressive disease; NC = no change; PR = partial response; CR = complete remission; NE = not evaluable.
‡No hematologic toxicity observed.

TABLE 2.—Treatment with adriamycin (37.5 mg/m²) and cyclophosphamide (500 mg/m²) every 3 weeks *

Patient No.	Age/ sex	Diagnosis, site	Site of metastases	Prior therapy	No. of courses	Total dose (mg)		Response
						Adria- mycin	Cyclophos- phamide	
1	68/F	Adenocarcinoma, uterus	Lung, vagina	RT	11	750	8000	CR (9+ mos); on maintenance with cyclophosphamide
2	68/F	Adenocarcinoma, uterus	Lung	Medroxyprogesterone	6+	320	4950	PR (4+ mos)†
3	66/F	Adenocarcinoma, uterus	Peritoneum	RT, medroxyprogesterone	5	370	4700	NC (3 mos); PD†
4	47/F	Adenocarcinoma, breast	Liver, bone, ascites	RT, 5-FU, prednisone, fluoxymesterone	3	180	2250	NE; liver size regression; ascites worse
5	65/M	Adenocarcinoma, lung	Lung, bone	RT	3	199	2625	PD
6	28/M	Adenocarcinoma, parotid	Lung	RT, oral cyclophosphamide + 5-FU + MTX + VCR	3	210	3500	PD
7	37/M	Undifferentiated carcinoma (? 1°)	Lung, pleura, axillary lymph node	RT	8+	370	3700	PR (5+ mos)
8	31/M	Embryonal carcinoma, retroperitoneal	Liver, lung	Actinomycin D + VCR + bleomycin	2	118	1800	PD
9	65/M	Transitional cell, renal pelvis	Bone	RT	7	400	5500	NC (3 mos); PD
10	19/F	Osteogenic sarcoma, jaw	Jaw, orbit	RT	3	135	1850	PD; adriamycin alone, 2 doses†
11	52/F	Leiomyosarcoma, uterus	Pelvis, lung	None	5	300	4000	NC (3 mos); sudden death
12	76/F	Rhabdomyosarcoma, leg	Lung, pleura	None	3	160	1800	PD†
13	72/M	Mesenchymoma, liver	Liver	None	3	180	2400	PD

* Abbreviations and symbols same as in table 1.
† No hematologic toxicity observed.

two dose levels of adriamycin explored are summarized in tables 1 and 2. The number of courses given and the total doses received are also shown. Because hematologic toxicity was observed in many of the patients on the low-dose adriamycin regimen who had received extensive prior radiotherapy, such patients were preferentially entered into this regimen. On the other hand, after the initial experience with four patients receiving the preceding regimen, previously untreated patients were given the higher dose of adriamycin plus 500 mg/m² of cyclophosphamide. The hematologic toxicity associated with both treatment regimens is shown in table 3 and consisted predominantly of leukopenia with the nadir occurring between Days 10 and 14. Thrombocytopenia was only exceptionally observed. No cumulative hematologic toxicity was apparent. Other toxicities at the lower dose of adriamycin included nausea (nine patients), vomiting (seven patients), alopecia (eight patients), and local irritation from the iv injection (one patient). At the higher dose of adriamycin, the frequency of similar toxicity was eight, six, nine, and one, respectively. Stomatitis was observed in one patient at this dose level. No patient manifested severe or life-threatening cardiotoxicity. Two patients were hospitalized because of fever at the higher dose of adriamycin.

One response (patient No. 6, table 1) was observed in the low-dose adriamycin regimen out of 13 evaluable patients; this response lasted for 6 months and consisted of regression of an extrapleural mass and pleural effusion in a patient with a papillary adenocarcinoma (subsequently determined to be of ovarian origin) who had been unsuccessfully treated with a single intrapleural dose of mechlorethamine. Two other patients (patient No. 8 with lung carcinoma, large cell undifferentiated type, and patient No. 5 with adenocarcinoma of the ovary) (table 1) had transient stabilization of disease with subjective amelioration for 2 and 3 months, respectively.

In the regimen including the higher dose of adriamycin (37.5 mg/m²) three objective responses were noted among 12 evaluable patients, and three additional patients had stabilization of disease with subjective improvement. Noteworthy were the three patients with adenocarcinoma of the uterus, one patient being maintained in CR for 9+ months, another with a PR of 4+ months, and a third with NC after 3 months but with persistent abdominal masses. A PR of 5+ months was also obtained in a patient with an undifferentiated carcinoma of the mediastinum associated with chylothorax. Three patients, one with a transitional cell carcinoma of the renal pelvis, one with an adenocarcinoma of the ovary, and one with a leiomyosarcoma of the uterus, may have benefited from the combination for periods of 3–4 months. The median amounts of adriamycin and cyclophosphamide given in this regimen were 210 and 3500 mg, respectively, contrasted with 77.5 and 1500 mg in the preceding regimen. Five patients receiving the lower dose regimen and four patients receiving the higher dose regimen experienced no hematologic toxicity during treatment.

TABLE 3.—Myelotoxicity in patients treated with adriamycin-cyclophosphamide combinations

Regimen	Dose (mg/m²)	No. of patients	Median nadir counts × 10^3 (range) on: Days 10–15* Wbc	Days 10–15* Platelets	Day 22† Wbc	Day 22† Platelets
I	Adriamycin, 25 Cyclophosphamide, 500	15	4.3 (1.5–14.1)	>250 (60–590)	5.5 (2.0–10.9)	>250 (250–620)
II	Adriamycin, 37.5 Cyclophosphamide, 500	13	2.9 (1.2–5.9)	182 (132–500)	4.9 (2.9–10.9)	224 (118–520)

* First two courses only for each patient.

† Course with lowest value represented for each patient.

DISCUSSION

Theoretical advantages of combination chemotherapy over single-drug treatment of cancer include true therapeutic synergism, a delay in the emergence of resistance to individual agents thus prolonging therapeutic effectiveness, the minimizing of individual drug toxicity (4), and in this particular combination a postponement of the risk of cardiotoxicity associated with the administration of adriamycin above 550 mg/m² (1). The activity of combinations of adriamycin and cyclophosphamide in animal tumors has not been reported; it would be relevant to these theoretical considerations whether the combination has less-than-additive, additive, or greater-than-additive toxicity in Skipper's L1210 leukemia model (4).

Possible disadvantages to be considered are the compounding of toxicity without enhancing therapeutic effects (greater-than-additive toxicity) and the inability to administer effective doses of either agent in tumors where one drug might be superior to the other. To minimize this last consideration, this adriamycin-cyclophosphamide study included types of malignancy against which either agent alone is expected to have some efficacy. In addition, lengthy prior exposure to alkylating agents was a criterion for ineligibility.

The results indicate that adriamycin and cyclophosphamide can be given safely in combination in a 3-week schedule at 50% of their individually tolerable doses. A dose of 37.5 mg/m² of adriamycin and 500 mg/m² of cyclophosphamide caused leukopenia in nine of 13 patients with the nadir occurring between Days 10 and 15 of the cycle and usually complete recovery by Day 22. Because some patients will not exhibit dose-limiting toxicity at these doses, further escalation appears possible after the initial course. Indeed, a number of therapeutic regimens in current clinical trial have combined these two drugs at similar doses in the treatment of breast cancer (5) or slightly higher doses with other agents including prednisone and vincristine (CHOP) in the treatment of malignant lymphomas (6) or vincristine and 5-(3,-3-dimethyl-1-triazeno) imidazole-4-carboxamide in the treatment of soft tissue sarcomas (7).

It cannot be stated from the present study whether therapeutic advantages resulted from the combination as opposed to single-agent treatment. The data do suggest that the combination including the higher dose of adriamycin was therapeutically useful since a beneficial effect was apparent in six of 12 evaluable patients including one who obtained CR and two who obtained PR. One PR was also observed in the low-dose adriamycin regimen.

The most notable therapeutic result was in the treatment of advanced endometrial carcinoma, a malignancy for which there is no generally effective chemotherapy at the present time. Three patients were treated, two after the failure of progesterone therapy. Patient No. 1 (table 2) had a cough and dyspnea due to pleural and pulmonary metastases from a uterine adenocarcinoma proven by pleural biopsy and dilation and curettage (D and C). A prompt symptomatic response within 2 weeks of the initial treatment was observed, and the patient entered a CR which is still ongoing 9 months after the start of therapy. Patient No. 2 (table 2) also had multiple pulmonary nodules secondary to endometrial adenocarcinoma proven by D and C. The patient was bedridden by dyspnea and required oxygen at a time when, after the failure of progesterone therapy, adriamycin-cyclophosphamide treatment was begun. Again, a prompt response was noted followed by complete absence of respiratory symptoms in the ensuing 4 months. Progressive regression of the pulmonary metastases, not as yet complete, has been documented on serial chest roentgenogram. Patient No. 3 (table 2) had, notwithstanding progestational therapy, a slowly progressive picture of intestinal obstruction secondary to extensive intra-abdominal metastatic uterine adenocarcinoma when treatment was initiated. Cessation of emesis and abdominal pain constituted a subjective response to therapy. No evidence of disease progression developed over a 3-month period, although abdominal tumor measurements did not change appreciably. During the 4th month, intestinal obstruction recurred and adriamycin-cyclophosphamide treatment was terminated. Thus, of three cases of advanced endo-

metrial cancer, one CR, one sustained PR, and one subjective response with no objective change for 3 months were recorded. This experience compares favorably with the inconsistent palliation previously reported with the use of chemotherapeutic agents, which include only rare instances of CRs (2). Cyclophosphamide alone (2) and adriamycin alone (8) have been among the most effective agents. However, because of their relative inefficacy, treatment of advanced endometrial cancer with these chemotherapeutic agents has only been considered after the failure of hormonal therapy, a modality that has produced a 30%–35% remission rate in the most favorable series (9). The preliminary results reported here do suggest that the combination of adriamycin and cyclophosphamide is worthy of further study in the treatment of this disease.

In the treatment of adenocarcinoma of the breast our experience was not favorable, but most patients received the low-dose adriamycin regimen, had had extensive prior treatment, and had far-advanced disease. Similarly, additional experience is needed at the higher dose level in patients with ovarian cancer and sarcomas to fully explore the usefulness of this combination. Lung cancer is another neoplasm where the combination might merit a more extensive evaluation in randomized studies versus the individual drugs given sequentially.

Whether the combination possesses therapeutic synergism cannot be stated at present. However, on the basis of these preliminary observations further phase II studies of the combination appear desirable in a variety of solid tumors, particularly in endometrial cancer.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053; dactinomycin

Adriamycin: NSC-123127

Bleomycin: NSC-125066

Chlorambucil: NSC-3088; CAS reg. No. 305-03-3; butyric acid, 4-[*p*-[bis(2-chloroethyl)amino]phenyl]-

CHOP: cyclophosphamide, adriamycin, vincristine (Oncovin), and prednisone

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate

5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide: NSC-45388; CAS reg. No. 4342-03-4; DIC

***d*-Tetrandrine:** NSC-77037

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU

Fluoxymesterone: NSC-12165; CAS reg. No. 76-43-7; androst-4-en-3-one, 9-fluoro-11β,17β-dihydroxy-17-methyl-

Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride; HN_2; nitrogen mustard

Medroxyprogesterone: NSC-26386; CAS reg. No. 71-58-9; pregn-4-ene-3,20-dione, 17-hydroxy-6α-methyl-, acetate; Depo-Provera

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-; MTX

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

Progesterone: NSC-9704; CAS reg. No. 57-83-0; pregna-4-ene-3,20-dione; progestin

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1); VCR

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ADDENDUM

At the XIth International Cancer Congress, Florence, Italy (Oct 20–26, 1974), I. Wodinsky et al presented data of therapeutic synergism of adriamycin-cyclophosphamide combinations in L1210 leukemia (Proceedings, vol 4, p 737); Lokich et al presented clinical data similar to ours (Proceedings, vol 1, p 369).

Orthostatic Hypotension During Combination Chemotherapy With Vincristine (NSC-67574) [1,2]

Joseph Aisner,[3] Howard D. Weiss,[3,4] Paul Chang,[3] and Peter H. Wiernik[3,5]

SUMMARY

Severe orthostatic hypotension developed in four patients with cancer during combination chemotherapy which included vincristine. We believe the orthostatic hypotension may be a manifestation of vincristine neurotoxicity. Milder degrees of orthostatic hypotension may be a relatively common complication of this chemotherapeutic agent.

[Cancer Chemother Rep 58:927–930, 1974]

The growing use of drugs in the treatment of neoplastic diseases has given rise to a myriad of potential iatrogenic complications, including various forms of neurologic dysfunction (1). We have recently encountered three patients with Hodgkin's disease in whom symptomatic orthostatic hypotension developed during MOPP [6] combination chemotherapy (2) and a fourth patient with Ewing's sarcoma in whom orthostatic hypotension developed during combination chemotherapy with cyclophosphamide [7] and vincristine. Orthostatic hypotension has been previously reported to occur only rarely during vincristine (3,4) or procarbazine (5) therapy.

CASE REPORTS

Patient No. 1.—A 24-year-old woman was found to have stage IIA Hodgkin's disease (6). She received radiotherapy to the involved and adjacent lymph nodes. Four weeks after the completion of radiation therapy she was given combination chemotherapy with MOPP (2). The doses of mechlorethamine and procarbazine were subsequently reduced to compensate for leukopenia. After three courses of MOPP the patient began to complain of light-headedness. Her blood pressure was 110/60 lying, 90/40 sitting, and 55/20 standing, with an increase in heart rate. Neurologic examination showed decreased deep tendon reflexes (DTRs) and absent ankle jerks. The patient was eating well and was not dehydrated. Vincristine therapy was discontinued. Six weeks later identical left hemiplagia and hemianesthesia developed twice lasting several hours, each time after the patient assumed an upright posture. Examination of the heart, lungs, and abdomen showed no abnormalities. Lumbar

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[2] Vincristine: CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1). Supplied by Cancer Therapy Evaluation, Division of Cancer Treatment (DCT), National Cancer Institute (NCI).

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[6] MOPP = mechlorethamine, vincristine (Oncovin), prednisone, and procarbazine.

Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2′-dichloro-*N*-methyl-, hydrochloride; nitrogen mustard.

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-.

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride.

[7] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

puncture, glucose, electrolytes, hematocrit, blood urea nitrogen (BUN), isotopic brain scan, electroencephalogram, electrocardiogram, and right carotid arteriogram were all within normal limits. The patient was kept at bedrest, and a body stocking helped alleviate the orthostatic pulse and blood pressure changes. She slowly resumed graded activity and after 2 months was able to tolerate physical activity without the body stocking. Chemotherapy was completed without additional vincristine. The patient remains in complete remission and without residual orthostatic complaints or changes.

Patient No. 2.—A 47-year-old man was found to have stage IA Hodgkin's disease. He was given radiotherapy to involved plus adjacent lymph nodes and 6 weeks later was given MOPP combination chemotherapy. The doses of procarbazine and mechlorethamine were decreased in subsequent doses due to pancytopenia, but vincristine was given at full dose. By the fifth course of drug therapy the patient had pronounced paresthesias and absent DTRs, and he complained of orthostatic light-headedness. His blood pressure was 100/60 lying, 90/50 sitting, and 70/40 standing, with an associated tachycardia. The patient was not dehydrated and electrolytes, glucose, hematocrit, and BUN were normal. Vincristine was discontinued and the patient experienced slow resolution of paresthesias and orthostatic complaints. No other cause for orthostatic hypotension was found. Three months after completion of chemotherapy all paresthesias were gone, reflexes returned to normal, and there were no blood pressure changes when he assumed an upright position. At present, the patient is asymptomatic and remains in complete remission of his Hodgkin's disease.

Patient No. 3.—A 20-year-old man was found to have stage IIB Hodgkin's disease. He was given radiotherapy to involved plus adjacent lymph nodes followed 6 weeks later by MOPP combination chemotherapy. He required dosage adjustments of the mechlorethamine and procarbazine after three courses due to leukopenia. After the fourth course of chemotherapy he began to complain of orthostatic light-headedness. Shortly after starting the fifth course of MOPP the patient had a syncopal episode. On examination his blood pressure was 110/60 lying, 90/40 sitting, and 75/40 standing. Neurologic examination disclosed mild symmetrical proximal muscle weakness, absent ankle jerks, and diminished DTRs. The patient had been eating well and was not dehydrated. The results of blood chemistry tests were within normal limits. No other cause for the orthostatic hypotension could be found. Other autonomic functions were apparently normal, and there were no bowel, bladder, or genital symptoms. Vincristine was discontinued and the paresthesias and orthostatic hypotension slowly resolved. The patient is currently in complete remission.

Patient No. 4.—A 14-year-old girl was found to have localized Ewing's sarcoma. She was given radiation therapy to her tumor with simultaneous weekly intravenous doses of vincristine (2 mg) and cyclophosphamide (500 mg/m^2). Constipation developed after the first two doses of vincristine and the third dose was omitted. The vincristine was restarted after the patient's bowel movements resumed and she received doses 4–6 on schedule. Three days after her sixth dose of vincristine, the patient had a near syncopal episode upon arising from bed. After resting flat for several hours she was able to tolerate a sitting position and was brought to the hospital. Physical examination showed the supine blood pressure to be 115/70 with a pulse of 96 and regular; standing blood pressure was 70/0 with a pulse of 140 and thready. She was afebrile and well hydrated. Examination of the heart, lungs, abdomen, and extremities showed no abnormalities. Neurologic examination disclosed hypoactive DTRs. Hematocrit, glucose, BUN, serum electrolytes, and stool guaiac were all within normal limits. Vincristine therapy was stopped and the patient's orthostatic hypotension slowly resolved.

DISCUSSION

In conventional doses, alkylating agents have not produced neurologic disorders (7), but both vincristine and procarbazine are potentially neurotoxic. Vincristine commonly produces peripheral neuropathy characterized by

decreased or absent DTRs, paresthesias, and weakness, with relatively little objective sensory loss (3,8,9). Electrophysiologic studies suggest that the pathophysiology of vincristine neuropathy is related to axonal degeneration (10–12). The incidence and severity of vincristine neuropathy are dose related (13,14) and neuropathy is usually reversible when the dosage is lowered or the drug discontinued (3).

Vincristine can produce autonomic nervous system dysfunction. Colicky abdominal pain and constipation occur in over one third of the patients treated (3,9). Other autonomic disturbances such as adynamic ileus, impotence, bladder atony, and urinary retention have been reported (9,15). Orthostatic hypotension has been previously described only as a rare complication (3,4). Orthostatic hypotension due to vincristine as opposed to other neurologic complications was not seen in any of the patients treated by Sandler et al (3) or by Holland et al (9).

Procarbazine inhibits monoamine oxidase (16) and can deplete plasma pyridoxine (17), both actions being potentially neurotoxic. Lethargy (18), personality disorders (19), myalgias, paresthesias, and decreased DTRs (5) have been reported during procarbazine therapy.

The four patients described in this paper all had orthostatic hypotension during combination chemotherapy with vincristine. Three of these patients also received procarbazine. In each case, the disorder resolved or is resolving after deletion of vincristine. None of these patients had urinary retention, anhidrosis, impotence, or other symptoms suggesting a generalized disorder of the autonomic nervous system, nor did any of them have other pre-existing disorders that predispose to orthostatic hypotension. Consequently, it is highly likely that the orthostatic hypotension was caused by the patients' chemotherapy. It is impossible to determine whether vincristine or procarbazine was mainly responsible for the symptoms in the three patients with Hodgkin's disease since both agents have been reported to occasionally cause orthostatic hypotension. It is interesting to note that these three patients were receiving the full dose of vincristine (2 mg/week) when the orthostatic hypotension began, whereas the procarbazine dosage had earlier been reduced due to leukopenia. The conventional signs of vincristine neurotoxicity were relatively mild in these cases and tended to resolve when the orthostatic hypotension resolved. Gottlieb and Cuttner (15) found that the severity of autonomic nervous system toxicity need not correlate with other manifestations of vincristine neurotoxicity, and there is no evidence from previous clinical trials to suggest that vincristine and procarbazine exert cumulative or synergistic neurotoxicity (2).

Carmichael et al (4) studied catecholamine metabolism and excretion in a patient with orthostatic hypotension related to vincristine administration. There was virtually no norepinephrine or normetanephrine excretion; epinephrine levels were normal and norepinephrine infusion showed normal end-organ responsiveness. Six weeks after vincristine discontinuation norepinephrine levels rose to normal accompanied by improvement in the hypotension. Norepinephrine excretion was also shown to be decreased in other patients receiving vincristine. Since the reflex arc was shown to be intact, a lesion of the postganglionic nerve was suggested. Furthermore, both norepinephrine and its metabolites were decreased with normal 3-methoxy-4-hydroxymandelic acid excretion, suggesting a norepinephrine storage or release problem in the nerve terminal. The exact mechanism of vincristine-induced orthostatic hypotension still needs to be elucidated.

The incidence of orthostatic hypotension during vincristine therapy is difficult to assess. Until the first case of orthostatic hypotension occurred (patient No. 1), we did not specifically look for this disorder. Since that index case, however, three additional severe cases (patients 2–4) became apparent within months, and we suspect that milder degrees of orthostatic hypotension may be more common than previously believed. Recognition of the association between chemotherapy and orthostatic hypotension may be especially important in monitoring older patients and patients receiving other hypotensive medications in order to prevent serious hypotensive episodes.

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Prevention of Cyclophosphamide (NSC-26271) Immunosuppression by Bacillus Calmette-Guérin [1,2]

Brief Reports and Preliminary Communications

David P. Houchens, Marilyn R. Gaston, Yvonne Kinney, and Abraham Goldin[3,4]

Studies in this laboratory have shown that pretreatment with bacillus Calmette-Guérin (BCG) is able to partially prevent immunosuppression by cyclophosphamide in Moloney sarcoma virus (MSV)-infected mice, as measured by the increase in median survival time (MST) and in the number of survivors (1). MSV has been shown to protect syngeneic mice against challenge with Moloney leukemia virus (MLV)-induced leukemias (2). It was therefore of interest to determine whether immunosuppression of mice with cyclophosphamide would interfere with the ability of MSV to protect against MLV-induced leukemia (LSTRA), and whether the prevention of immunosuppression by BCG would restore the ability of MSV to protect against MLV-induced tumor.

Refrigerated viable BCG was kindly supplied by the Pasteur Institute, Paris, France. Each ampule contained 25 mg of BCG in a volume of 0.5 ml of 0.9% NaCl. This was further diluted in 0.9% NaCl for injection. All injections were 1 mg/mouse (2×10^6 viable organisms) in a volume of 0.2 ml by the intraperitoneal (ip) route. Male BALB/c mice weighing 20–25 g were used in the experiment. These were obtained from the Drug Research and Development production colonies. A pool of virus (Lot No. MSV-A-1) was prepared from MSV-infected muscle tissue of BALB/c mice by the procedure of Moloney (3). For these tests, 0.5 ml of a 1:50 dilution was injected intramuscularly into the right hind leg.

LSTRA, a tumor in BALB/c mice originally induced by MLV, was maintained in ascites form by ip passage in male BALB/c mice. For our experiment, the tumor cells were counted and the concentration adjusted for inoculation. Experimental mice were challenged with 10^4 tumor cells by the subcutaneous (sc) route. Cyclophosphamide was dissolved in 0.9% NaCl and injected ip at a dose of 210 mg/kg.

Animals were injected with BCG. Two weeks later some of these animals and other animals that had not received BCG were immunosuppressed with cyclophosphamide. Twenty-four hours later these animals received MSV. At various time intervals (Days 7, 14, 21, 35, and 80), mice were randomly selected from each test group and from a control group of untreated animals. These animals were challenged sc with 10^4 viable LSTRA cells and observed for 30 days.

Table 1 presents the data from the experiment. Normal mice challenged with LSTRA died with an MST range of 11–13 days (group 8). There were no survivors.

Inoculation of BCG and/or cyclophosphamide had no effect on MST or the number of survivors in non-MSV-injected mice that were challenged with LSTRA (groups 5–7). When mice were inoculated with MSV and challenged at the various intervals with LSTRA, there were significant increases in the MST and the number of survivors (group 4). When cyclophosphamide was given 1 day before MSV (group 3), the MST was reduced to the same range as in the animals receiving LSTRA

[1] Received June 4, 1974; revised July 17, 1974; accepted Aug 22, 1974.

[2] Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,-2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan.

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TABLE 1.—Effect of BCG on immune response in BALB/c mice challenged with LSTRA tumor after immunosuppression and/or immunization with MSV

				No. of days after MSV that LSTRA (10^4 cells sc) was given—													
				7			14			21			35			80		
Group	Treated with BCG*	CY†	MSV‡	MST (days)	ILS§ (%)	No. of 30-day survivors/ total treated (%)	MST (days)	ILS (%)	No. of 30-day survivors/ total treated (%)	MST (days)	ILS (%)	No. of 30-day survivors/ total treated (%)	MST (days)	ILS (%)	No. of 30-day survivors/ total treated (%)	MST (days)	ILS (%)	No. of 30-day survivors/ total treated (%)
1	+	+	+	20	74	2/8 (25)	>30	>161	4/8 (50)	>30	>130	4/8 (50)	>30	>161	6/8 (75)	>30	>172	5/8 (63)
2	+	−	+	>30	>161	4/8 (50)	>30	>161	3/4 (75)	>30	>130	6/6 (100)	>30	>161	6/6 (100)	>30	>172	7/8 (87)
3	−	+	+	12	4	0/8 (0)	15	30	3/12 (25)	15	15	1/8 (12)	ND¶	—	—	ND¶	—	—
4	−	−	+	22.5	96	2/8 (25)	>30	>161	5/8 (62)	>30	>130	5/8 (62)	>30	>161	5/7 (71)	>30	>172	5/8 (63)
5	+	+	−	12	4	0/8 (0)	12	4	0/8 (0)	13.5	4	1/6 (17)	12.0	4	0/6 (0)	11.5	14	0/8 (0)
6	+	−	−	12	4	0/8 (0)	12	4	0/8 (0)	12.0	0	0/8 (0)	12.5	9	0/6 (0)	11.5	14	0/8 (0)
7	−	+	−	11	0	0/8 (0)	12	4	0/8 (0)	14.0	8	0/8 (0)	12.0	4	0/6 (0)	11.0	0	0/8 (0)
8	−	−	−	11.5	—	0/8 (0)	11.5	—	0/8 (0)	13.0	—	0/8 (0)	11.5	—	0/8 (0)	11.0	—	0/8 (0)

*2×10^6 viable organisms administered ip 14 days before MSV.

†CY = cyclophosphamide. 210 mg/kg administered ip 1 day before MSV.

‡0.05 ml of 1:50 dilution of virus administered im in the right hind leg.

§ILS = increase in lifespan (% above controls).

¶ND = not determined. All recipient animals were dead with progressively growing sarcoma at this time.

alone (group 8). There was, however, a moderate increase in MST and some survivors at the 14-day interval. There were no recipient animals available in group 3 for tumor challenge on Days 35 or 80 because all animals designated for these groups had died with progressively growing sarcoma. Fefer (4) has demonstrated that mice immunosuppressed with cyclophosphamide and injected with MSV die with progressively growing tumors. The extent to which animals in group 3 died of progressively growing sarcoma or LSTRA tumor has not been clarified. All of the animals in this group died with large LSTRA tumors. However, at the time of death, 23 of 28 animals challenged at the three time intervals in this group also had palpable sarcomas.

When BCG was injected prior to the administration of cyclophosphamide and MSV and followed by LSTRA (group 1), the MST and number of survivors were similar to those observed for the animals receiving only MSV plus LSTRA (group 4). The immunosuppressive effect of cyclophosphamide seen in group 3 was reduced in group 1 as indicated by a significant increase in lifespan of group 1 mice. Group 2 mice, receiving BCG, MSV, and LSTRA, had a moderately higher percentage of survivors for each challenge interval than the mice that received MSV and LSTRA (group 4), although the differences were not significant.

The current experiment provides confirmation of the previous report from this laboratory on the effect of preadministration of BCG in immunosuppressed mice that are challenged with MSV (1). Animals immunosuppressed with cyclophosphamide showed progressive MSV tumor growth resulting in death prior to the scheduled time of LSTRA challenge (group 3, Days 35 and 80). However, when BCG administration preceded cyclophosphamide, MSV tumors grew and regressed (group 1, Days 35 and 80). Additional strong evidence that BCG prevents the effects of immunosuppression is provided by the maintenance of the immune system as measured by the response to the immunization with MSV and subsequent challenge with LSTRA. The immunity, once established, was maintained for more than 80 days after virus inoculation.

The nature of the reduction by BCG of cyclophosphamide immunosuppression has not been determined. The possibility that BCG might interfere with the activation of the drug is suggested by the report of Farquhar (5) that drug-metabolizing enzyme activities in BCG-treated mice are reduced considerably.

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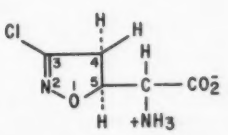
A New Antitumor Agent, (αS,4S,5R)-α-Amino-3-chloro-4-hydroxy-4,5-dihydro-5-isoxazoleacetic Acid (NSC-176324): Preliminary Evaluation Against L1210 Mouse Leukemia In Vivo [1,2,3]

Brief Reports and Preliminary Communications

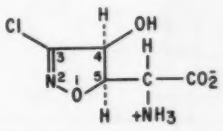
D. G. Martin, L. J. Haňka, and G. L. Neil [4,5,6]

Recently, we reported (1) that a novel antitumor antimetabolite, (αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (NSC-163501; U-42,126) (fig 1), was active against L1210 leukemia in mice. This agent, initially produced in minute amounts by fermentation of *Streptomyces sviceus* (2), was isolated and purified by ion-exchange chromatography, partition chromatography, and crystallization (3). In the course of isolating additional NSC-163501, mother liquors and less active chromatography fractions from an early fermentation were recycled by ion-exchange chromatography on Amberlite IR 45 (hydroxide cycle). The active eluates were evaporated, crystallized, and recrystallized several times to afford additional NSC-163501. Efforts to dissolve the residue obtained by evaporating the mother liquors in the phases of *n*-butanol:benzene:methanol:water (2:1:1:1) in preparation for partition chromatography afforded a suspension. The solid was collected, washed with methanol, and recrystallized from water, affording a highly crystalline sample melting with decomposition at 165°C. Spectral and analytic data indicated that the new amino acid was closely related to NSC-163501 with one of the methylene protons at C-4 replaced with a hydroxyl group (4). The hydroxylated amino acid, unlike NSC-163501, shows a tendency to associate with up to 1 equivalent of water which can be removed by drying under reduced pressure, but may be reabsorbed from atmospheric moisture.

X-ray crystallography confirmed the structure and rigorously established the absolute configuration of this new amino acid as (αS,4S,5R)-α-amino-3-chloro-4-hydroxy-4,5-dihydro-5-isoxazoleacetic acid (NSC-176324) (fig 1) (4). Although NSC-176324 and NSC-163501 exhibit similar chromatographic behavior on many paper chromatography and thin-layer systems, solvent systems based on methyl ethyl ketone, acetone, and water nicely resolve them on both paper and silica gel plates. Chromatograms are readily visualized with ninhydrin spray. Resolution of the amino acids on silica gel columns together with greatly improved fermentation titers allowed preparation of relatively large quantities of both NSC-163501 and NSC-176324 for biologic evaluation (5). Successive processing of 16,000 liters of clarified fermentation broth with Dowex 50 (hydrogen cycle) and then Amberlite IR 45 (hydroxide cycle) ion-exchange chromatography afforded a mixture of the two agents. A relatively efficient resolution on silica gel afforded readily crystallized fractions of each



NSC-163501
U-42,126



NSC-176324
U-43,795

FIGURE 1.—Chemical structures of NSC-163501 and NSC-176324.

[1] Received June 26, 1974; accepted July 19, 1974.

[2] Supported in part by contracts NO1-CM-33707 and NO1-CM-43753 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[3] NSC-176324: U-43,795.

[4] Cancer Research, The Upjohn Company, Kalamazoo, Mich.

[5] The technical assistance of A. E. Berger, T. F. DeKoning, K. Ebi, R. Garza, D. R. Horsfall, and S. L. Kuentzel is gratefully acknowledged.

[6] *Reprint requests to:* Dr. Gary L. Neil, Cancer Research, The Upjohn Company, Kalamazoo, Mich 49001.

component, yielding (after recrystallization) over 150 g of each pure antitumor agent (5).

Biologic Evaluation

When antibacterial activity was determined using a previously described (1) disc-plate assay employing an organism sensitive to NSC-163501 (*Bacillus subtilis* UC-902 growing in a synthetic medium), NSC-176324 was less than 0.1% as active as NSC-163501. This relative lack of in vitro antibacterial activity of NSC-176324 made bioautography of mixtures of it and NSC-163501 difficult.

When assayed for its ability to inhibit the growth of L1210 cells in culture (6), NSC-176324 was approximately five times less potent than NSC-163501. ID50 values (concentrations required to inhibit 3-day growth by 50%) were 0.58 and 0.12 μg/ml for NSC-176324 and NSC-163501 respectively.

NSC-176324 is approximately four to ten times less potent than NSC-163501 (on the schedule employed) in the treatment of L1210 leukemic mice (table 1). Significant therapeutic effects (%ILS >25) were observed at doses of 3.13 and 0.78 mg/kg/day of NSC-176324 and NSC-163501 respectively. Although NSC-176324 is not as potent as NSC-163501 in the treatment of L1210 leukemic mice, the optimum results achieved with the two agents are roughly comparable. Neither agent appears to offer a marked advantage over the other with respect to therapeutic ratio. For comparable therapeutic effects (%ILS), weight change was also similar.

Further detailed evaluation of the biochemical and pharmacologic effects of NSC-176324 and NSC-163501 is in progress. Of particular interest is the as yet unexplained dichotomy between the activity ratios in mammalian and bacterial systems. Preliminary biochemical mechanism of action studies suggesting that both agents are potent glutamine antagonists are described elsewhere (8).

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TABLE 1.—Comparison of the antitumor effect in L1210 leukemic mice of NSC-163501 and NSC-176324*

Daily dose† (mg/kg)	NSC-176324 Survival (days) Range	NSC-176324 Survival (days) Mean ± SD‡	NSC-176324 % ILS §	NSC-176324 Average weight change ¶ (g/mouse)	NSC-163501 Survival (days) Range	NSC-163501 Survival (days) Mean ± SD‡	NSC-163501 %ILS §	NSC-163501 Average weight change ¶ (g/mouse)
100	9–16	11.3 ± 2.3	28	–1.1				
50	11–18	14.2 ± 2.8	61	–0.9				
25	13–15	14.1 ± 1.0	59	0.0	5–8	7.3 ± 0.5	Toxic	–3.3
12.5	12–14	12.8 ± 0.8	45	–0.1	6–7	6.5 ± 0.6	Toxic	–4.1
6.25	12–16	12.7 ± 1.6	44	+0.4	5–12	8.8 ± 2.4	0	–3.3
3.13	10–12	11.2 ± 0.7	27	+0.7	9–14	11.8 ± 2.0	34	–1.4
1.56	8–11	9.8 ± 1.2	11	+0.6	11–17	13.2 ± 2.1	50	+0.2
0.78					10–12	11.5 ± 0.8	31	0.0
0.39					9–11	9.8 ± 1.0	11	+1.4
Control	7–10	8.8 ± 1.3	0	+0.9	7–10	8.8 ± 1.3	0	+0.9

* Antitumor testing (control 0072LE) was performed by methods described previously (1) according to protocols devised by the Division of Cancer Treatment, National Cancer Institute (7). Six female BDF_1 (C57BL/6 × DBA/2) mice per group (30 for controls) were inoculated ip on Day 0 with 1×10^5 L1210 cells/mouse.

† Treatment was ip daily, Days 1–9. Agents were administered in 0.5 ml 0.9% aqueous NaCl.

‡ Mean survivals and standard deviations (SD) were calculated.

§ Percent increase in lifespan (%ILS) was calculated from mean survivals of treated and control groups.

¶ Calculated from average weights on Days 1 and 5. Day 1 weights were 17.0–18.6 g/mouse.

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Calusterone (NSC-88536): A Poor Substitute for Fluoxymesterone (NSC-12165) in the Treatment of Advanced Breast Cancer[1,2,3]

Brief Reports and Preliminary Communications

G. Falkson, J. J. van Dyk, E. B. van Eden, A. M. van der Merwe, and H. C. Falkson[4,5]

Calusterone is a weakly androgenic synthetic steroid synthesized by The Upjohn Company.[6] Phase I clinical studies were conducted by Gordon et al (1,2) who reported objective regression in 14 of 22 women treated with calusterone; the only side effect was a weak androgen effect with inconstant sulfobromophthalein retention. An editorial in the *Journal of the American Medical Association* (3) stated that results in 102 patients treated with calusterone justified the opinion that it is "the most useful steroid now under study for the treatment of this disease." Because of the excellent response rates and the low toxicity reported with calusterone, a clinical trial was undertaken in Pretoria in 1972. Prior to completion of our trial, Goldenberg et al (4) and Lemkin et al (5) reported much less favorable results with calusterone.

MATERIALS AND METHODS

Fifty-nine patients with advanced metastatic cancer of the breast were included in the study. The metastatic sites were as follows: 22 skin, 21 soft tissue, 36 bone, ten liver, and seven lung. Patients with central nervous system (CNS) metastases were not included. There were two groups: group I consisted of 21 patients with metastatic disease who had previously received intravenous (iv) cyclophosphamide[7] after oophorectomy but whose disease was again progressive. They continued to receive oral maintenance therapy with cyclophosphamide (1.5 mg/kg/day × 5 consecutive days/week) throughout the study. The patients in group I were randomized at the start of the study to receive either calusterone (200 mg/day) or fluoxymesterone (30 mg/day). Group II consisted of 38 patients who had received no prior treatment for their metastatic disease. These patients were randomly allocated to either calusterone (200 mg/day) plus cyclophosphamide or fluoxymesterone (30 mg/day) plus cyclophosphamide. The median age of the patients, distribution of metastases, and performance status in the calusterone groups as compared to the fluoxymesterone groups were similar.

RESULTS

Therapeutic Effects

Group I.—Of the ten patients randomized to calusterone there were no complete remissions (CR) or partial remissions (PR); four patients improved (IMP), two showed no change (NC), and four had progression of disease (PD). Of the 11 patients randomized to fluoxymesterone there were also no remissions; four had IMP, four had NC, and three had PD. Therefore, no remissions were observed in the 21 patients in group I.

Group II.—Three of 18 patients treated with

[1] Received Dec 14, 1973; revised May 28, 1974; accepted June 21, 1974.

[2] Supported in part by a grant from the National Cancer Association of South Africa.

[3] Calusterone: CAS reg. No. 17021-26-0; testosterone, 7β,17-dimethyl-. Synthesized by The Upjohn Company, Kalamazoo, Mich.

Fluoxymesterone: CAS reg. No. 76-43-7; androst-4-en-3-one, 9-fluoro-11β,17β-dihydroxy-17-methyl-.

[4] Department of Cancer Chemotherapy, H. F. Verwoerd Hospital and the University of Pretoria, South Africa.

[5] *Reprint requests to:* Geoffrey Falkson, MD, Department of Cancer Chemotherapy, H. F. Verwoerd Hospital and the University of Pretoria, Pretoria, South Africa.

[6] Clinical brochure: U22,550, 17β,17-dimethyltestosterone, Jan 1972. Prepared by The Upjohn Company, Kalamazoo, Mich.

[7] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

calusterone plus cyclophosphamide had good responses (no CR, three PR, five IMP, three NC, and seven PD). Ten of 20 patients treated with fluoxymesterone plus cyclophosphamide had good responses (three CR, seven PR, four IMP, three NC, and three PD).

Calusterone was used in the treatment of a total of 28 patients and some benefit was observed in 12 (43%); fluoxymesterone was used in the treatment of 31 patients and was of benefit to 18 (58%).

Side Effects

Virilization.—Virilization occurred in 13 of 28 patients treated with calusterone and in 14 of 31 patients treated with fluoxymesterone. The duration of treatment bears a relationship to the development of virilization. Because of better response rates, fluoxymesterone was often used for a much longer period than calusterone.

Liver function.—No changes in liver function were ascribed to fluoxymesterone. Of the 28 patients treated with calusterone five had a decrease in albumin, seven had increases in alkaline phosphatase, five had increases in serum glutamic oxaloacetic transaminase, and two had increases in bilirubin determinations ascribed to calusterone. The liver became palpable in four patients. No deaths were ascribed to hepatic toxicity, and liver changes were reversed when calusterone was discontinued except in one patient in whom fatty changes and centrilobular necrosis were found at postmortem examination.

Fluid retention.—Peripheral edema, with or without swelling of face or hands, occurred in five of 31 patients who received fluoxymesterone and in 12 of 28 patients who received calusterone. Pulmonary edema was not seen in patients receiving fluoxymesterone but occurred in five of those patients receiving calusterone. This toxic effect was considered to be a possible contributing cause of death, and in two patients this theory was confirmed at postmortem examination.

Headache and malaise.—Headache and malaise were reported by only three patients receiving fluoxymesterone but were seen in 16 patients receiving calusterone. In these patients symptoms were often severe. Neurologic examinations and scintiscans were necessary to exclude CNS metastases.

Hypercalcemia.—Hypercalcemia occurred in only one patient treated with fluoxymesterone but was documented in seven patients receiving calusterone. Calcium values up to 18 mg/100 ml were recorded. Other patients exhibited side effects suspected of being due to hypercalcemia.

Nausea and hemopoietic suppression.—Patients receiving calusterone had a much lower nausea tolerance to cyclophosphamide, and the dose of cyclophosphamide that caused hemopoietic suppression was less in the group treated with calusterone.

DISCUSSION AND CONCLUSIONS

Recent advances in the treatment of metastatic cancer of the breast have come from the earlier use of cytostatic agents and from the combination of hormone treatment and cytostatic treatment (6–8). Optimistic results reported for calusterone (1–3) prompted this trial. However, this study failed to confirm the high response rate for calusterone and also showed that the agent is associated with considerably more side effects than is a standard androgen. Furthermore, the tolerance of patients with advanced breast cancer to cyclophosphamide was considerably better when fluoxymesterone was used than when calusterone was used. It was initially planned that this study would include a large number of patients in order to evaluate drug combinations adequately in various stratifications of breast cancer (age, postmenopausal status, metastases, and disease-free interval at the start of treatment). The poor tolerance to and high toxicity of calusterone resulted in the termination of this study sooner than anticipated.

The importance of toxic effects from hormone treatment must not be underestimated and it is felt that patients placed on this trial may have been deprived of the benefit of earlier use of cytostatic combinations.

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Combination Chemotherapy With Methotrexate (NSC-740), Adriamycin (NSC-123127), Cyclophosphamide (NSC-26271), and 5-Fluorouracil (NSC-19893) for Adult Solid Tumors [1,2,3]

Ronald L. Stephens and Barth Hoogstraten [4,5]

Adriamycin, the *Streptomyces peucetius*-derived antibiotic, has an antineoplastic spectrum unrivaled by any of the other new agents that have been introduced since the advent of cyclophosphamide in the early 1960s. It is not only effective in the more sensitive cancers such as leukemia and lymphoma, but it has also demonstrated activity as a single agent in more resistant cancers such as sarcoma (1), bladder (2), and lung (3). Adriamycin has shown excellent activity in the tumor types showing a more intermediate responsiveness to chemotherapy, particularly in breast cancer where it is nearly twice as effective as other compounds (4).

The antimetabolite 5-fluorouracil (5-FU), the folate antagonist methotrexate, and the alkylating agent cyclophosphamide have a long history of usefulness in the treatment of cancer (5). Together with adriamycin, they form a potentially useful combination of drugs with different biochemical and kinetic cytotoxicities.

This study was undertaken to establish a tolerable dose level of these four drugs in combination, anticipating a broader phase II trial in specific tumors.

MATERIALS AND METHODS

The patient population consisted of nine women and seven men whose ages ranged from 41 to 70 years (mean 50 years). Patients were eligible for this study if they were not responsive to more standard treatments and had not previously received adriamycin or more than one of the other agents.

Patients were free of overt heart disease, had a normal electrocardiogram, a blood urea nitrogen level less than 25 mg/100 ml, and a bilirubin level less than 1.1 mg/100 ml. The baseline white blood cell (wbc) count had to exceed 4000 cells/mm^3 and the platelet count had to be greater than 150,000 platelets/mm^3.

The initial treatment regimen consisted of adriamycin, 40 mg/m^2 given intravenously (iv) every 3 weeks; 5-FU, 100 mg/m^2 given iv weekly; methotrexate, 10 mg/m^2 given iv weekly; and cyclophosphamide, 100 mg/day given orally. There were two exceptions to this regimen where the initial doses were reduced because of bone marrow hypocellularity. Subsequently, these patients tolerated a full regimen. Since an additional myelosuppressive drug was added (adriamycin) the initial dose of 5-FU was considerably lower than customarily utilized in combination with methotrexate and cyclophosphamide. A planned escalation of 5-FU was deferred for this group of patients due to levels of leukopenia obtained with the above regimen.

[1] Received Mar 28, 1974; revised June 28, 1974; accepted July 22, 1974.

[2] Supported in part by Public Health Service grant CA-12644 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare, and the Wittman-Glick Foundation.

[3] Methotrexate: CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

Cyclophosphamide: CAS reg. No. 6055–19–2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

5-Fluorouracil: CAS reg. No. 51-21-8; 5-FU.

The compounds used in this study were supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, NCI.

[4] Division of Oncology, Department of Medicine, University of Kansas Medical Center, Kansas City.

[5] *Reprint requests to*: Ronald L. Stephens, MD, Division of Oncology, Department of Medicine, University of Kansas Medical Center, Rainbow Blvd at 39th St, Kansas City, Kan 66103.

RESULTS

Most patients experienced manageable nausea and vomiting within a few hours of adriamycin administration and alopecia developed in most. Stomatitis occurred in four patients and, unlike the stomatitis of high-dose methotrexate, was never dose limiting. Hematologic toxic effects consisted mainly of leukopenia. All 16 patients began with a normal wbc level and all had counts that dropped by at least 3000 cells/mm^3. One patient's wbc level remained normal, one had a wbc nadir below 4000, six had levels below 3000, five had levels below 2000, and three had a drop to less than 500 cells/mm^3. Two of the last three patients required hospitalization and intensive antibiotic therapy for infection, but no fatal toxic effects were observed.

Generally, the time to wbc nadir was predictable in all patients. It consistently occurred 2 weeks after a dose of adriamycin, although the nadir did not always occur within the first cycle of therapy. Thrombocytopenia was moderately severe in only one patient (32,000 cells/mm^3) and was almost certainly due to the use of methotrexate in this patient who had suboptimal renal function.

Although the primary purpose of this study was to ascertain a tolerable dose level for this four-drug combination, the activity of this combination by primary site is interesting. Three of five patients with breast cancer had objective response, with healing of bone lesions in two patients and a more than 50% shrinkage of subcutaneous tumor masses in one patient. One patient had a subjective response with dramatically decreased bone pain and weight gain. On Day 99 the fifth patient with breast cancer was judged to have progressive disease as measured by the increasing size of lytic bone lesions.

Seven patients with lung cancer were treated with this combination. One of four patients with the squamous-cell variety had an excellent partial response, with shrinkage of the primary tumor observed on the chest roentgenogram and complete flattening of a convex skull metastasis. This response was apparent by Day 51 of the study and has been sustained for 280+ days. The other three patients with the squamous-cell variety had no response, as did two patients with adenocarcinoma and one with large-cell anaplastic lung cancer. One patient with renal-cell carcinoma, two with bladder cancer, and one with colon cancer had no improvement.

DISCUSSION

The four-drug combination of methotrexate, adriamycin, cyclophosphamide, and 5-FU resulted in a wbc nadir of 2000 cells/mm^3 or less in eight of 16 patients. The platelet-sparing property of adriamycin has been noted by others (2) and, with one exception, this was clearly the case in our patients. It is anticipated that a higher dose of 5-FU can be given, particularly in previously untreated patients, and this will be the subject of a future report.

Our patient population is too small to draw serious conclusions about the phase II activity of this combination. However, it is clear that this four-drug program has excellent activity in breast cancer, where three of five patients had meaningful objective responses, and where one of the other two patients has sustained an excellent symptomatic improvement. These four drugs will be the subject of future investigations in breast cancer. One of four patients with the squamous-cell variety had an excellent objective and subjective response.

It would be reasonable to continue investigating this combination in a phase II study of lung cancer. Although 5-FU appears to be of additional benefit when used in combination therapy for breast cancer, it is doubtful that it is of benefit when used in combination therapy for lung cancer. In addition, since it is myelosuppressive, this agent may actually detract from future exploration of adriamycin combinations in lung cancer.

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Clinical Trial of BCNU (NSC-409962) Combined With Vincristine (NSC-67574) in Disseminated Gastrointestinal Cancer and Other Neoplasms [1,2,3]

Brief Reports and Preliminary Communications

David C. Stolinsky, Reginald P. Pugh, Richard A. Bohannon, Donald L. Bogdon, and Joseph R. Bateman [4,5]

Employed separately, both BCNU and vincristine have demonstrated activity against a variety of malignant neoplasms (1). Encouraging results were reported by Moon (2) who combined these agents in the treatment of patients with melanoma. Accordingly, the Western Cancer Study Group undertook a phase II trial of BCNU plus vincristine. Emphasis was placed on gastrointestinal cancer because of a report by Moertel et al (3) on the use of BCNU alone, and because of the need for an effective regimen for patients whose tumors are resistant to 5-fluorouracil (5–FU).[6]

METHODS

Patient Selection

Patients with measurable malignant tumors not amenable to surgical or radiation therapy were included in the study. Patients whose tumors were resistant to BCNU or vincristine were ineligible. Patients were classified as a "poor risk" if they had any of the following characteristics: a hypocellular marrow; extensive prior radiation therapy; marrow involvement by tumor; or poor tolerance to other agents in the past. Of the 273 patients in the study, 201 had received prior chemotherapy. Of the 95 patients with carcinoma of the stomach or colon, 94 had received prior treatment with 5-FU.

Clinical Evaluation

Pretreatment examination of patients included: physical examination with bidimensional tumor measurements; complete blood cell count (cbc) including platelets; urinalysis; blood urea nitrogen, uric acid, bilirubin, alkaline phosphatase, and glutamic pyruvic transaminase determinations; bone marrow aspiration; and chest roentgenogram. Physical examination and cbc were repeated weekly; serum chemical determinations and roentgeno-

[1] Received Apr 22, 1974; revised July 1, 1974; accepted July 18, 1974.

[2] Supported in part by Public Health Service grant CA-05186 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] BCNU: CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-.

Vincristine: CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

The compounds used in this study were supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, NCI.

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[6] 5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU.

grams were repeated every 2-4 weeks. In the assessment of response, the products of the bidimensional measurements of each tumor were added together.

Dose Schedule

Patients considered to be good risks received 75 mg/m² of BCNU intravenously (iv) on each of 2 consecutive days, while patients classified as poor risks received 50 mg/m² of BCNU on each of 2 consecutive days. BCNU administration was repeated every 4 weeks. All patients received vincristine (1 mg/m²) iv every 2 weeks. BCNU was withheld in the presence of significant leukopenia or thrombocytopenia, and vincristine was withheld in the presence of significant neuropathy. Patients were removed from the study if they failed to attain at least a minimal response within 4 weeks after receiving a BCNU dose that produced significant toxic effects.

RESULTS

All 273 patients who were treated were evaluated for response and for toxic effects. However, 31 patients died of disease within 4 weeks after the start of therapy.

Responses

A minimal response was defined as a reduction in tumor size of 25%–49% for at least 1 month. A partial response was defined as a reduction in tumor size of 50% or more for at least 1 month. A complete response was defined as the disappearance of all manifestations of disease for at least 1 month (table 1). Responses began at a median of 17 days after the start of therapy (range, 7–140 days), at a median BCNU dose of 150 mg/m² (range, 80–600 mg/m²), and at a median vincristine dose of 1.3 mg/m² (range, 1–8 mg/m²). Responses lasted a median of 2.5 months (range, 1–23 months).

TABLE 1.—Experience with BCNU plus vincristine in the treatment of 273 patients

Tumor	No. of patients studied	No. of patients with— Minimal response	Partial response	Complete response
Esophagus	3	2		
Stomach	10			
Colon, rectum	85	3	7	1
Hepatoma	1			
Biliary tree	3			
Pancreas	8	1		
Nasopharynx	4			
Larynx	2			
Lung, oat cell	8	2	1	
Lung, anaplastic	9		1	
Lung, squamous cell	6			
Lung, adenocarcinoma	12		1	
Parotid	2			
Thymoma	1			
Kidney	8			
Bladder	2			
Prostate	3		1	
Testis	3			
Breast	10			
Ovary	7			
Cervix	2			
Fallopian tube	3			
Unknown site, anaplastic	12		2	
Unknown site, adenocarcinoma	12			
Neuroblastoma	1			
Adrenal cortex	1			
Astrocytoma	1			

TABLE 1.—Experience with BCNU plus vincristine in the treatment of 273 patients—Continued

Tumor	No. of patients studied	No. of patients with— Minimal response	Partial response	Complete response
Melanoma	8		2	
Bone, giant cell	1			
Osteosarcoma	6			
Synovial sarcoma	1			
Spindle cell sarcoma	4			
Liposarcoma	3			
Myxosarcoma	2			
Fibrosarcoma	4			
Leiomyosarcoma	11		1	
Rhabdomyosarcoma	3		1	
Ewing's sarcoma	1			
Kaposi's sarcoma	1			
Chordoma	2			
Anaplastic sarcoma	5		1	
Mesothelioma	2			
Totals	273	8	18	1

Toxic Effects

Leukopenia was classified as mild (3000–3900 cells/mm³), moderate (2000–2900 cells/mm³), severe (1000–1900 cells/mm³), or extreme (< 1000 cells/mm³). Thrombocytopenia was classified as mild (100,000–148,000 platelets/mm³), moderate (60,000–99,000 platelets/mm³), severe (35,000–59,000 platelets/mm³), and extreme (< 35,000 platelets/mm³) (table 2). Three patients died with infectious complications during a period of leukopenia, and one died of hemorrhage during a period of thrombocytopenia. The isolated instance of hemorrhagic cystitis was apparently related to therapy; it recurred when therapy was restarted and cleared when therapy was discontinued.

DISCUSSION

Although responses were noted in a variety of malignant neoplasms, the overall response rate of 7.0% was disappointing. The response rate for patients with carcinoma of the stomach and colon, including partial and complete responses, was only 8.4%, indicating that this regimen is ineffective in patients with gastrointestinal carcinoma that is resistant to treatment with 5-FU. Additional patients with melanoma and lymphoma were evaluated

TABLE 2.—Toxic effects of BCNU plus vincristine in the treatment of 273 patients

Toxic effect	Degree of toxicity (No. of patients) Mild	Moderate	Severe	Extreme
Leukopenia	40	24	11	2
Thrombocytopenia	38	35	15	9
Nausea, vomiting	65	45	8	
Constipation	22			
Alopecia	32	5		
Neuropathy	59	26	1	
Stomatitis	1	1		
Hemorrhagic cystitis	1			
Dermatitis			1	
Irritation at injection site	3			

in a separate study. Further trial of BCNU combined with vincristine in the treatment of other neoplasms was abandoned.

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100% Oxygen Inhalation and 5-Fluorouracil (NSC-19893) Therapy for Large Bowel Carcinoma[1,2,3]

Charles G. Moertel, Allan J. Schutt, R. J. Reitemeier, and R. G. Hahn[4,5]

It has been well established that the cells of many solid tumors are hypoxic because of an inadequate capillary blood supply. The fact that the metabolic processes of these cells are operating under conditions of oxygen deprivation could be postulated as a cause of insensitivity to therapeutic efforts designed to attack these metabolic processes. It has been clearly established in cell culture and animal model systems that increased oxygenation increases the susceptibility of tumor cells to ionizing radiation. To date, however, attempts to capitalize on this oxygen effect in the clinical radiation therapy setting have not produced conclusive results. Laboratory evidence justifying an assumption that oxygen concentration may produce alterations in the response of tumor cells to cytotoxic drugs is fragmentary at best. Few attempts have been made to approach this problem clinically. Nevertheless, it is a relatively easy question to address with clinical research approaches that presumably would hold little hazard for the patient.

This study was designed to evaluate, by double-blind method, the effects of 100% oxygen inhalation on toxicity and chemotherapeutic response to 5-fluorouracil (5-FU) therapy for large bowel carcinoma.

METHOD

All of the 149 patients who were chosen for this study had histologically confirmed metastatic unresectable adenocarcinoma of the large bowel. All were ambulatory outpatients who had been maintaining a reasonable state of nutrition prior to entry. No patient had had previous therapy with either 5-FU or 5-fluoro-2′-deoxyuridine,[6] and none had had radiation or chemotherapy of any kind for at least 1 month prior to entry. Each patient had a measurable area of known malignant disease to serve as an objective indicator of response to therapy. Discrete lesions observed on chest roentgenograms, cutaneous lesions, or palpable masses clearly measurable with a ruler or caliper were considered acceptable. Lesions demonstrable only by contrast roentgenography or lesions for which size could only be estimated, eg, pelvic masses, were not acceptable. If hepatomegaly due to cancer was used as an indicator lesion, it was required that hepatic metastasis be proven by biopsy and that a clearly defined liver edge extend at least 5 cm below the costal margin on quiet respiration.

These patients were treated with 5-FU which was administered on a randomized basis either by rapid intravenous injection at a dosage of 13.5 mg/kg/day for 5 consecutive days or by 2-hour infusion at a dosage of 20-25 mg/kg/day for 5 consecutive days. The results of this comparison have been previously reported (1). In essence, each method produced comparable toxicity and comparable therapeutic response.

Also on a randomized basis, patients in each 5-FU regimen were assigned either to receive 100% oxygen or atmospheric air through a demand-type mask. The tanks were shrouded and neither patient nor investigator was aware of the inhalation therapy assignment. Inhalation therapy was begun 10 minutes before initiation of 5-FU therapy and continued for 2 hours thereafter.

All patients who could tolerate further treatment received a second course of therapy at 5 weeks. Dosage for this course was readjusted in order to produce definite but clinically toler-

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[3] 5-Fluorouracil: CAS reg. No. 51-21-8; 5-FU. Supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, NCI.

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[6] 5-Fluoro-2′-deoxyuridine: NSC-27640; 5-FUDR.

able levels of toxicity. The same type of inhalation therapy for which the patient was randomized for the first course of treatment was also repeated during the second course of treatment.

An evaluation of the therapeutic results was conducted 10 weeks after the first course of therapy was initiated. An objective response was declared if there was a reduction of at least 50% in the product of the largest perpendicular diameters of the most clearly measurable area of known malignant disease (chosen prior to entry as a primary indicator lesion). If hepatomegaly was the indicator lesion, it was required that there be a reduction of at least 30% in the sum of measurements below the xyphoid and below each costal margin at the midclavicular lines without any deterioration of liver function tests (serum glutamic oxaloacetic transaminase, alkaline phosphatase, and serum bilirubin). If there was an increase in size of other areas of malignant disease, or if new areas of malignant disease appeared, the result was considered a treatment failure.

RESULTS

In assessing the results of this study, all patients were considered evaluable if they were randomized to the study and received their first dose of 5-FU. If patients died before completion of their planned therapy or became too sick to continue therapy, they were tabulated as treatment failures.

Toxic Effects

The gastrointestinal and mucocutaneous toxic effects experienced by the two patient groups during their first course of therapy are shown in table 1 and the frequency and severity of leukopenia are shown in table 2. The small differences do not approach statistical significance.

Therapeutic Response

The objective response rates observed at 10 weeks are recorded in table 3. Again, any differences are minor and statistically insignificant.

DISCUSSION

Using our methods, inhalation of 100% oxygen during 5-FU therapy was associated with only slight changes in the toxicity pattern and therapeutic response. These changes can probably be attributed to chance alone; but even if they were real, they would hardly justify the increased cost and nuisance of oxygen inhalation. It is, of course, possible that different approaches to this problem (such as the addition of carbon dioxide to oxygen, the use of hyperbaric oxygen chambers, or the use of more radiomimetic chemotherapeutic agents) could produce a more advantageous effect. The singularly negative results of this study, however, offer scant encouragement for such pursuits.

TABLE 1.—Gastrointestinal and mucocutaneous toxic effects

Toxic effects	Percent of patients with toxic reaction	
	Oxygen (total, 75 patients)	Atmospheric air (total, 74 patients)
Vomiting	34	34
Stomatitis	47	36
Diarrhea	18	12
Dermatitis	7	7
Alopecia	5	5

TABLE 2.—Hematologic toxic effects: leukopenia

Degree of leukopenia (cells/mm³)	Percent of patients with leukopenia	
	Oxygen (total, 75 patients)	Atmospheric air (total, 74 patients)
< 4000	58	54
< 2000	20	16
< 1000	9	7

TABLE 3.—Objective response at 2 months

Disease status	Percent of patients with indicated status	
	Oxygen (total, 75 patients)	Atmospheric air (total, 74 patients)
Regression	13	11
Stable	32	27
Progression	55	62

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ANNOUNCEMENTS

Third International Symposium on Detection and Prevention of Cancer

New York City, New York, April 26 to May 1, 1976

Americana Hotel

This Symposium is designed to promote an international exchange of information on cancer control through basic research, prevention, and detection.

The Scientific Program of the Symposium will include recent experimental findings and clinical studies. Their role in the detection and prevention of cancer will be discussed and evaluated in different session formats including: lectures by leading authorities on progress in various disciplines; panels of invited guest speakers for discussion of multidisciplinary topics on the role of basic cancer research in the prevention of malignant tumors and their detection during asymptomatic stages, epidemiology, environmental and occupational carcinogens, interaction between etiologic factors, early biologic manifestations of tumors, potentially malignant alterations, molecular oncology, cell kinetics, cytogenetics, and radiation; and symposia which include *selected proffered papers* on recent advances in detection and diagnosis of subclinical and early clinical stages of tumors in specific sites (breast, lung, gastrointestinal tract, bladder, female genital system), and various aspects of general population screening. Adequate opportunities for discussion are planned.

Plans have been made for a closing panel devoted to the summation of preceding sessions, scientific and commercial exhibits, and advanced workshops on various phases of cancer prevention, detection, and diagnosis (immediately after the symposium).

The official language of the Symposium will be English, with simultaneous translation into French and Spanish.

Please address inquiries to: H. E. Nieburgs, MD, Secretary General, Third International Symposium on Detection and Prevention of Cancer, Mount Sinai School of Medicine of the City University of New York, 5th Ave and 100th St, New York, NY 10029.

Cancer Chemotherapy Abstracts

Commencing with Volume 15, *Cancer Chemotherapy Abstracts*, prepared under the auspices of the National Cancer Institute, has been expanded and published under a new title, *Cancer Therapy Abstracts*.

Cancer Therapy Abstracts will constitute the world's most comprehensive key to the literature of all modes of cancer treatment. In addition to the world's literature on cancer chemotherapy, it will cover publications on cancer-related surgery, radiotherapy, and immunotherapy. Volume 15 and succeeding volumes will consist of 12 fully indexed issues, plus cumulative annual author and subject (agents, human tumors, experimental tumors, general subjects) indexes. *Cancer Therapy Abstracts* will cover approximately 6000 items per year, doubling the size of *Cancer Chemotherapy Abstracts*. The subscription price for Volume 15, *Cancer Therapy Abstracts*, is $65.00 domestic USA, and $75.00 foreign. The first issue of Volume 15 is currently available. In addition to printed form, *Cancer Therapy Abstracts* will be available, for an additional charge, on computer tape on a cumulative annual basis. The cumulative tapes for Volume 15 will be available in August 1975. The tape record will contain full descriptive cataloging, abstract or annotation, and index tracings for each item. Cumulative annual

computer tapes will also be available for Volumes 13 and 14 of *Cancer Chemotherapy Abstracts*. These will be ready in October 1974.

Prepaid subscriptions to *Cancer Therapy Abstracts*, and inquiries regarding the tape service, should be forwarded to: Information Resources Press, 2100 M Street, NW, Suite 316, Washington, DC 20037.

Symposium on "The Delayed Consequences of Cancer Therapy: Proven and Potential"

Orlando, Florida, January 9–11, 1975
Sheraton Olympic Villas, 6700 Sand Lake Rd

This conference is sponsored by the Cancer Clinical Investigations Review Committee of the National Cancer Institute. The Program Chairman is Giulio J. D'Angio, MD. The following topics will be covered: Basic Mechanisms of Injury; Late Effects of Chemotherapy; Late Effects of Radiation Therapy; Late Effects of Combined Chemotherapy and Radiation Therapy on Organ Function, Immune Competence, Oncogenesis, etc; and Epidemiology.

Participants: G. Canellos, G. Casarett, W. Creasey, G. D'Angio, A. Evans, Z. Fuks, A. Gilladoga, E. Gehan, T. Hall, C. Harris, J. Harris, F. Hendrickson, G. Hutchison, N. Jaffe, R. Johnson, T. King, M. Lane, C. Lushbaugh, A. Meadows, M. Mendelsohn, P. Morris-Jones, M. Nesbit, J. Nickson, R. Parker, I. Penn, T. Philips, G. Ross, U. Saffiotti, C. Shellabarger, H. Tagnon, M. Tefft, L. Wasserman, D. White, J. Wilson, and R. Withers.

For further information please write to: Mrs. Jeannette Steinbraker, Rm 10A03, Westwood Bldg, National Institutes of Health, Bethesda, Md 20014.

Oncology Placement Service

The American Society of Clinical Oncology's Clearing House of Oncologic Opportunities is accepting inquiries from applicants concerning staff positions and oncology fellowships, as well as announcements of openings for such applicants. The service is open to members and non-members of ASCO. Please direct all inquiries and announcements to Michael J. Brennan, MD, American Society of Clinical Oncology, 110 E Warren, Detroit, Mich 48201.

Eleventh Symposium on Biomathematics and Computer Science in the Life Sciences

Houston, Texas, April 3–5, 1975

The University of Texas Health Science Center at Houston, Division of Continuing Education, and The Region V of IEEE, announce the Eleventh Symposium on Biomathematics and Computer Science in the Life Sciences which will be held April 3–5, 1975, at the Marriott Motor Hotel, Houston, Texas, under the direction of General Chairman, Dr. Stuart O. Zimmerman.

The program will emphasize mathematical, statistical, bioengineering, and computing applications in biology and medicine.

Papers of an interdisciplinary nature will be welcomed by the Program Committee, as will appropriate papers on education and communication. Prior to January 1, 1975, an abstract of the paper must be submitted to the Program Chairman, Dr. Birger Jansson, The National Large Bowel Cancer Project, The University of Texas Cancer System at Houston, M.D. Anderson Hospital and Tumor Institute, Prudential Building, Room 1801, Houston, Tex 77025.

Concurrently, an exhibit for instruments, computer components, etc, relevant to biomathematics and bioengineering will be arranged. Exhibitors are welcome to direct their questions to the Exhibition Chairman, Dr. A. S. Badger, Mandrel Products, 6909 Southwest Freeway, PO Box 36827, Houston, Tex 77036.

For further information write: Dr. Stuart O. Zimmerman, c/o Office of the Director, The University of Texas Health Science Center at Houston, Division of Continuing Education, PO Box 20367, Houston, Tex 77025.

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